School of Veterinary and Life Sciences

Honours Thesis

Determination of the utility of oligonucleotide sequences specifically designed to disrupt the interaction of microRNA-494 with the *PROS1* mRNA transcript

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Declaration:

I declare this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution.

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Alyssa Rocchi

Acknowledgments

At the start of this year I didn’t think I was ever going to be able to find my way around the lab let alone do all the work I have accomplished. What started off as stressful ended up becoming second nature to me. I could not have done this without the constant support from everyone around me. I would like to firstly like to thank my parents for their support during my undergrad, without that I wouldn’t be where I am today. I’d also like to thank my best friends, who knew exactly what I was going through at all times this year.

The next group of people I would like to thank are the teams at the Western Australian Centre for Thrombosis and Haemostasis (WACTH) and Perth Blood Institute (PBI), Dr. Quintin Hughes, Dr. Jim Tiao, Dr. Jasmine Tay, Grace Gilmore, Sue Powell, Giuliana D'Aulerio and of course Prof. Ross Baker. From day one I felt welcome and part of the team and never felt like I couldn’t ask questions or ask for help, which I needed quite a lot of. I’d also like to thank my fellow honours students Michael and Matthew, doing similar lab work resulting in similar mishaps led to us encouraging each other when we felt like everything was going wrong. Additionally, I’d like to thank PhD student, Munik for all the help in and out of the lab.

A special mention goes to my supervisor, Dr. Quintin Hughes. He was able to remain calm through all of my lab problems and was always available to answer my questions no matter how many or how stupid they might have been. I am very grateful for the opportunity I was given with this honours project and I hope I have done it justice. Overall, I am grateful for this learning experience and thankful for everyone who was around me and gave me support throughout this year.

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Abbreviations

α	Alpha
β	Beta
2'OMe	2' O methyl phosphorothioate oligonucleotide
APC	Activated Protein C
ASO	Antisense oligonucleotide
C4BP	Complement component 4 binding protein
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
EGF	Epidermal growth factor
FDP	Fibrin degradation product
GP	Glycoprotein (i.e. GPIb)
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
kDa	Kilodalton
LNA	Locked chain nucleic acid (oligonucleotide)
miRNA	Micro-ribonucleic acid
miR-494	Micro-ribonucleic acid 494
MRE	Micro-ribonucleic recognition element
mRNA	Messenger-ribonucleic acid
PABP	Poly(A)-binding protein
PC	Protein C
PS	Protein S
RISC	Ribonucleic acid induced silencing complex
RNA	Ribonucleic acid

siRNA	Small interfering-ribonucleic acid
TAFI	Thrombin activatable fibrinolysis inhibitor
TF	Tissue factor
T_m	Melting Temperature
t-PA	Tissue plasminogen activator
UTR	Untranslated region
VTE	Venous thromboembolism
vWF	von Willebrand factor

Abstract

Micro-ribonucleic acids (miRNA) are non-coding RNA molecules that function as negative regulators of cellular processes, for example gene expression, by binding to sites found within the 3' untranslated region (UTR) sequence of target messenger RNAs (mRNAs). A previous study identified miR-494 as a direct regulator of the *PROS1* gene expression of Protein S (PS), with three functional binding sites being classified with the 3'UTR sequence. This regulation is a natural physiological process; however, it can become problematic for individuals with a hereditary PS deficiency. This current study hypothesised that the use of specifically targeted antisense oligonucleotide (ASO) sequences to the three functional binding sites found within *PROS1* mRNA 3'UTR can disrupt, or block the binding of miR-494 resulting in a subsequent increased expression of PS. HuH-7 cells were co-transfected with miR-494 and ASOs and luciferase assay and mRNA analysis performed. Luciferase assay results showed a proof of principle when utilizing ASOs to block the interaction of miR-494 with the PROS1 3`UTR. The results were trending towards reversal for selected ASOs, however, not statistically significant. Subsequent mRNA analysis, did however demonstrate a clear significant reversal of the previously observed effect of miR-494 on the levels of PROS1 mRNA transcripts in the presence of the ASOs, when compared to miR-494 alone. Interestingly, further analysis suggested that key modifications designed to increase the stability and affinity of the ASOs was important for the potency of the blocking effects. Overall, this project provides preliminary evidence towards negating the regulation miR-494 has on the *PROS1* 3'UTR. These results are significant for individuals with a heterozygous mutation that is causative for a protein deficiency disease, as further testing can lead to a novel therapeutic agent that can bypass existing mutations, resulting in the increased expression of the remaining functional allele.

Chapter 1: Introduction

1.1: Introduction

Hereditary deficiencies of natural anticoagulants such as antithrombin (AT), Protein C (PC) and Protein S (PS) contribute to a high-risk factor for venous thromboembolism (VTE) (De Stefano et al. 2006). The risk factor for these individuals is a 2- to 11- fold increase in comparison with those who do not have a deficiency (Lipe and Ornstein 2011). VTE is a chronic disease that is associated with considerable morbidity and mortality. In the long term, approximately 30% of patients have a recurrence within 10 years (Di Nisio, van Es, and Büller 2016).

The coagulation cascade and fibrinolytic system along with natural anticoagulants are all tightly regulated processes that are in place to prevent unnecessary blood loss and excessive clot formation. However, there is increasing evidence that suggests additional molecules, in particular micro-ribonucleic acids, are operating in the regulation of blood clotting and the development of thrombotic diseases.

1.2: Haemostasis

1.2.1. Introduction to Haemostasis

Haemostasis is a tightly regulated physiological process; this regulation is in place to ensure the protection of the vascular system from major blood loss as a result of injury (Gale 2011; Nesheim 2003). Regulation occurs through the presence of pro- and anti-coagulation factors. These factors ensure blood loss is kept at a minimum at the site of injury and fluidity of blood is maintained elsewhere, respectively (Nesheim 2003). Haemostatic balance is

controlled through four key components – vascular endothelium, platelets, the coagulation cascade and fibrinolysis. These components remain under tight regulation as any imbalance can result in thrombotic or haemorrhagic events that are associated with morbidity and mortality (Austin 2017). Haemostasis consists of two phases known as the primary haemostasis and secondary haemostasis.

1.2.2. Primary Haemostasis: Platelet activation and aggregation

The first step in primary haemostasis involves platelets, that are moving with sheer force through a vessel, interacting and tethering to damaged endothelium. This is achieved through the platelet surface glycoprotein, GPIb, binding with exposed subendothelial von Willebrand factor (vWF). Once platelets are tethered to the damaged endothelium, a platelet surface glycoprotein receptor, GPIIb-IIIa, changes its shape to allow binding of the glycoprotein GPIIb-IIIa and fibrinogen. This allows stronger adhesion and begins platelet activation. Through the binding of various molecules to specific receptors, the platelets become activated, resulting in additional platelets arriving and adhering to the already adherent platelets. This process results in the aggregation of platelets which rapidly form a unstable microthrombus (Austin 2017).

1.2.3. Secondary Haemostasis: Coagulation cascade

There are 12 factors involved in the coagulation cascade, each with a specific function that ultimately results in a stable fibrin clot (Table 1.1.). The Tissue Factor (TF) pathway of coagulation occurs when there is damage to the endothelium lining a blood vessel. TF is released from the damaged

endothelium, once released it binds to and activates factor VII → VIIa. The then complexed TF-VIIa can activate both factors IX and X (FIXa and FXa; Fig. 1.1). This is the initiation phase and is followed by the propagation phase. The initial FXa produced is able to convert small amounts of prothrombin to thrombin itself, this thrombin activates FV→FVa. During the propagation phase FXa combines with its co-factor Va to convert more prothrombin to thrombin, generating a sufficient amount to induce platelet aggregation and activation of additional co-factors V and VIII (FVa and FVIIIa). The increased amplification of FXa is achieved through the action of FIXa and FVIIIa. The increase in FXa results in continued conversion of prothrombin to thrombin, consequently converting fibrinogen to fibrin and activating factor XIII (FXIIIa) which is able to cross-link the fibrin to form a stable clot (Austin 2017).

Table 1. 1. List of factors involved in the coagulation cascade and their respective functions (Palta, Saroa, and Palta 2014).

Factor	Name	Function
I	Fibrinogen	Converted to fibrin for clot formation
II	Prothrombin	Activation of Factors I, V, VII, XI, XIII and PC and platelets
III	Tissue Factor	Cofactor for activated Factor VII
IV	Calcium Ions	Facilitates activated factors binding to phospholipids
V	Labile Factor	Complexes with Factor X to activate Prothrombin to Thrombin
VII	Stable Factor	Activates Factors IX and X
VIII	Antihemophilic Factor	Cofactor for Factor IX in the activation of Factor X
IX	Christmas Factor	Activates Factor X
X	Stuart-Prower Factor	Complexes with Factor V to activate Prothrombin to Thrombin
XI	Plasma Thromboplastin	Activates Factor IX
XII	Hageman Factor	Activates Factors XI and VII
XIII	Fibrin Stabilising Factor	Crosslinks Fibrin

1.2.4. Fibrinolysis

The coagulation cascade and fibrinolysis simultaneously respond to the release of an activator from damaged endothelium; in fibrinolysis, the activator is tissue plasminogen activator (t-PA) that converts plasminogen into the active enzyme, plasmin. Plasmin is the main enzyme that is activated in this pathway, it degrades fibrin strands resulting in the production of fibrin degradation products (FDPs). Thrombin complexed to thrombomodulin is a regulator of fibrinolysis; it is able to activate and form tissue activatable plasminogen inhibitor (TAFI) which is a negative regulator of the conversion of plasminogen to plasmin (Fig. 1.2.) (Nesheim 2003).

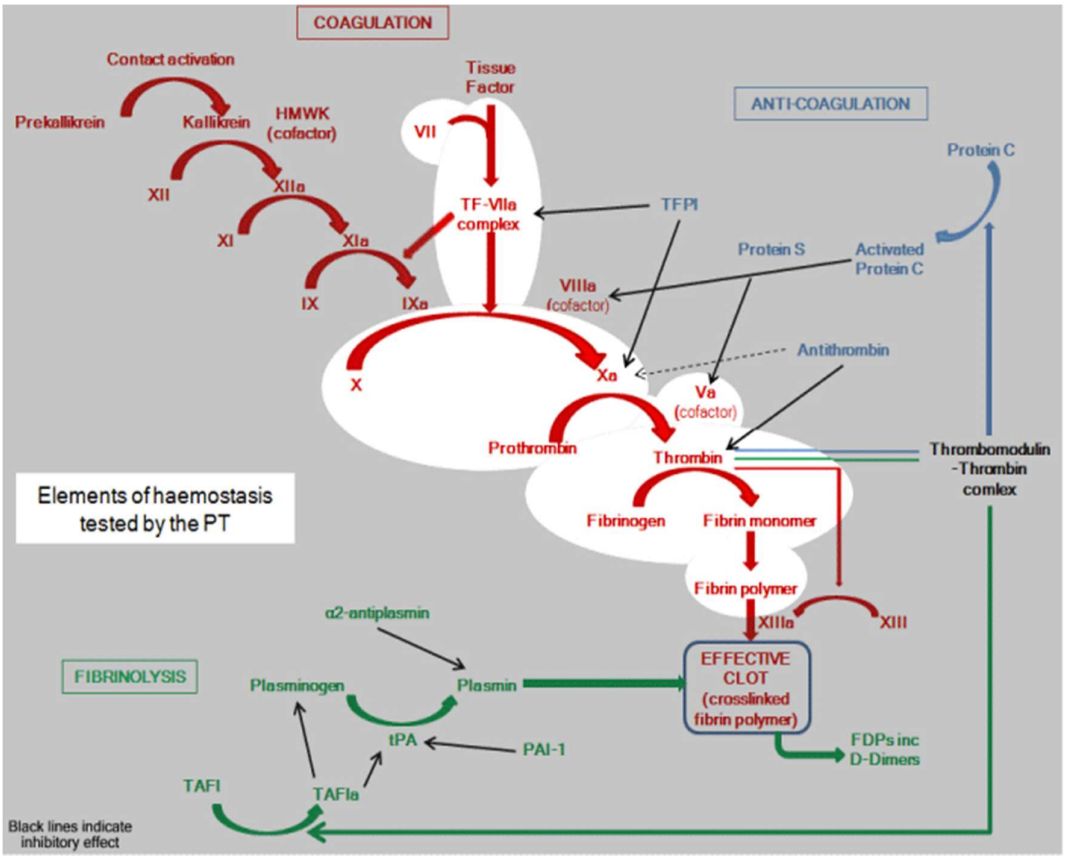


Figure 1.1: Schematic representation of the coagulation cascade. Including its regulatory proteins (Incorporated 2012)

1.2.5. The Anticoagulant System

Under normal physiological conditions, the maintenance of blood fluidity favours the natural anticoagulant system (Mac Hale, Nathan, and D'Ambra 1997). The interaction between thrombomodulin, PC and PS is one pathway that affects the coagulation cascade, helping to maintain blood fluidity (Esmon 2003). This anticoagulation pathway is initiated by the binding of thrombomodulin complexed with thrombin, onto PC. Once PC is activated (APC), it is able to form a complex with free PS, consequently enabling it to function as an anticoagulant (Esmon 2000; Esmon 2003) (Fig. 1.2). APC is a poor anticoagulant on its own, but in the presence of PS, its activity is greatly enhanced (García de Frutos et al. 2007), resulting in an increased ability for APC to degrade membrane- bound FVa and FVIIIa (Andersson et al. 2010; Giri et al. 1998). This demonstrates how the efficiency of the PC anticoagulation pathway is dependent on the presence of free PS in circulation. Accordingly, PS will be the focus in this project.

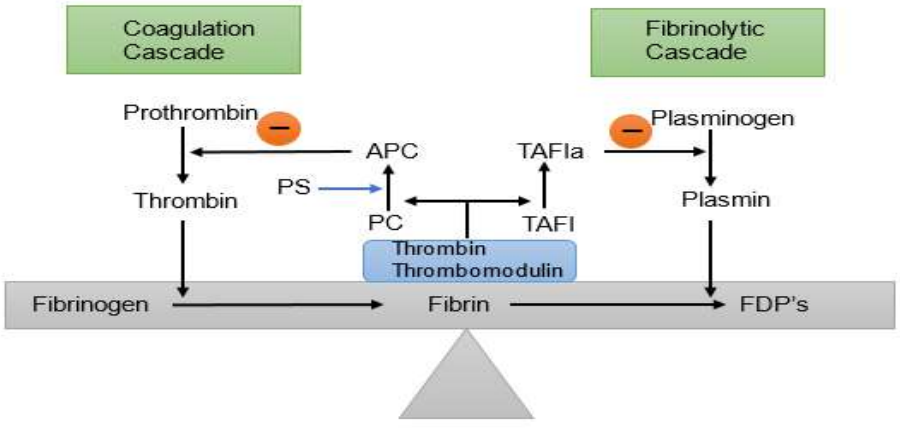


Figure 1.2. The balance between the coagulation cascade and fibrinolysis. Regulated by the thrombin/thrombomodulin complex (Nesheim 2003).

1.3: Protein S

PS is a vitamin K-dependent plasma glycoprotein with a circulating concentration within human plasma measuring approximately 20-25 mg/l (García de Frutos et al. 2007; Taniguchi et al. 2016). PS is expressed by the gene *PROS1* and synthesised mainly by hepatocytes in humans, however, synthesis also occurs through vascular endothelial cells, monocytes and megakaryocytes (Mac Hale, Nathan, and D'Ambra 1997; Taniguchi et al. 2016; Rezende, Simmonds, and Lane 2004). The mature, circulating PS has a structure that consists of an N-terminal Gla-domain, a thrombin-sensitive region (TSR), four epidermal growth factor domains (EGF) and a C-terminal sex hormone-binding globulin region (Fig. 1.3.) (Rezende, Simmonds, and Lane 2004; Mac Hale, Nathan, and D'Ambra 1997). Dahlbäck, Hildebrand, and Malm (1990) characterised a large assembly of monoclonal antibodies against PS. This was done to determine the regions available on PS that are important for its APC cofactor activity. It was determined that the sites for APC interaction were located in the TSR and the first EGF-domain (Dahlbäck, Hildebrand, and Malm 1990; He et al. 1998). Likewise, the Gla-domain was determined to be important for the binding of PS to membrane-bound FVa and FVIIIa, which is essential for the functional activity of PS (Giri et al. 1998).Within circulation PS has two forms: free PS and PS complexed to the human C4b-binding protein (PS-C4BP). The availability of these two forms under normal conditions are 40% and 60% respectively (Heeb et al. 2004). Human C4BP is a regulator of free PS and by extension, the PC pathway. C4BP is composed of six to seven alpha (α)-chains, with approximately 80% of the circulating protein containing a single beta (β)-

chain that enables binding to PS via the sex hormone-binding globulin region (Van De Poel, Meijers, and Bouma 1999; Rezende, Simmonds, and Lane 2004; Maurissen et al. 2008). Binding of this protein to PS forms the PS-C4BP complex, resulting in a decreased level of free PS and therefore a decreased cofactor function of PS for APC (Van De Poel, Meijers, and Bouma 1999). As mentioned, 60% of PS is complexed with C4BP under normal conditions, this is due to its high affinity and 1:1 stoichiometry (Rezende, Simmonds, and Lane 2004). During an acute-phase inflammatory response, the concentration of C4BP can increase up to four times (Van De Poel, Meijers, and Bouma 1999), however the concentration of free PS remains stable due to a mechanism that ensures differential regulation of the expression of the α- and β-chains, with β-chain expression being reduced during this inflammatory response (Van De Poel, Meijers, and Bouma 1999; García de Frutos et al. 1994).

While PS has been shown to have an efficient effect within the APC anticoagulation pathway, there have been studies showing PS as having anticoagulation activity independent of APC (Heeb et al. 1993; Heeb et al. 2004). This independent anticoagulation is a feature that occurs through free PS and PS-C4BP directly binding and inhibiting FVa, FVIIIa and FXa in the coagulation cascade and its ability to compete with pro-coagulation factors on the surface of phospholipids (Heeb et al. 1994; D'Angelo and D'Angelo 2008). Additionally, by binding to FVa, PS is able to directly inhibit prothrombinase activity, competing for binding with prothrombin, effectively (Heeb et al. 1993).

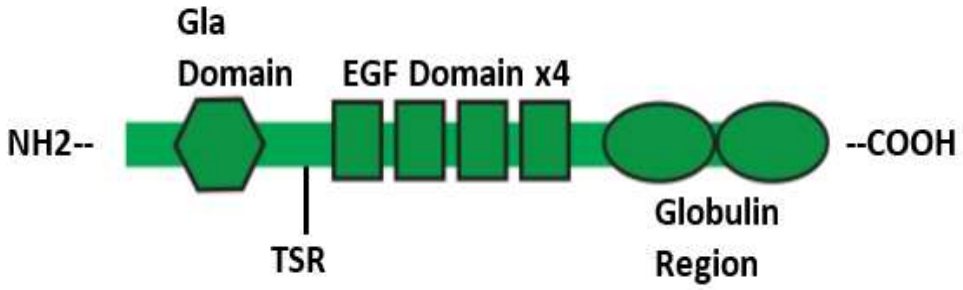


Figure 1.3. Schematic representation of the structure of Protein S. Featuring an N-terminal Gla-domain, a thrombin sensitive region, 4 EGF domains and a C-terminal globulin region (van der Meer, van der Poll, and van 't Veer 2014)

1.3.1. Protein S Deficiency

The *PROS1* gene is located on chromosome 3 at position 3p11.1-q11.2 (Gandrille et al. 2000). There are two copies of the gene, with *PROS1* being the functional copy and *PROS2* being the transcriptionally inactive pseudogene sharing approximately 97% homology to *PROS1*, however it lacks exon 1 (García de Frutos et al. 2007; ten Kate and Van der Meer 2008). Several studies have performed screening for mutations within *PROS1*, resulting in the characterisation of more than 200 different mutations that result in the loss of function of the gene (ten Kate and Van der Meer 2008; Pintao et al. 2009). The mutations that have been found to be associated with a PS deficiency have mostly been point mutations or short deletions/ insertions. Large genomic deletions within *PROS1* have been reported in 3-6% of cases (Caspers et al. 2012).

PS deficiency can be either inherited or acquired. Acquired PS deficiency can be oestrogen mediated, come about as a result of various pathological states or through warfarin therapy (D'Angelo and D'Angelo 2008).

Pathological states include individuals with disseminated intravascular coagulation (DIC), liver disease (D'Angelo et al. 1988) and nephrotic syndrome (Vigano-D'Angelo et al. 1987). There have also been cases reported showing the development of autoimmune PS deficiency resulting from chickenpox in children (D'Angelo et al. 1993). This project will however be focussing on inherited PS deficiency. The hereditary form of PS deficiency is autosomal dominant (ten Kate and Van der Meer 2008). Three types of hereditary PS deficiency have been determined in relation to the levels of bound and free PS in the plasma as well as its functional activity, these are type I, type II and type III (Hurtado et al. 2008). Type I, a quantitative defect, is characterised by decreased levels of both bound and free PS; type II, a qualitative defect, is characterised by normal levels of both bound and free PS but reduced anticoagulant activity; and type III, a mild quantitative defect, is characterised by decreased levels of free PS, while the bound levels are normal (Hurtado et al. 2008; Brouwer et al. 2005). It has been shown that most of the mutations associated with *PROS1* result in the quantitative defects of type I and III and are distributed along the entire gene, with no region displaying a higher frequency of mutation compared to another (García de Frutos et al. 2007). The type II defect is usually caused by a missense mutation which affects the EGF-domains, therefore altering the ability of APC binding (García de Frutos et al. 1994; Pintao et al. 2009). A defect within the *PROS1* gene is always heterozygous or in rare cases double heterozygous, as the homozygous form is lethal *in-utero* or can lead to life-threatening thrombotic complications soon after birth (Mustafa, Pabinger, and Mannhalter 1995; Khan and Dickerman 2006).

The hereditary deficiency of PS is a known risk factor for VTE (Brouwer et al. 2009), with approximately 50% of individuals with the heterozygous defect suffering a thrombotic event before the age of 50 (Anderson and Spencer 2003). Engesser et al. (1987) conducted a study to analyse the clinical manifestations present with a hereditary PS deficiency. Of the 71 individuals assessed 39 had experienced VTE, with 27 of those being recurrent. There were also individuals that experienced different combinations of deep vein thrombosis (DVT), pulmonary embolism and superficial thrombophlebitis (Engesser et al. 1987). Hereditary PS deficiency is an independent risk factor for VTE, however there are acquired risk factors that can increase the occurrence of VTE in these individuals. Risk factors include prior VTE occurrence, the age of the individual, with individuals >40yrs at greater risk, major surgery, immobility, pregnancy and the use of oral contraceptives (Anderson and Spencer 2003).

1.4: Regulation of Gene Expression

Regulation of gene expression is an important process undertaken by cells, to control the quantity and specificity of resultant proteins. During development, expression of certain genes are switched on or off as a way to give rise to diverse and specific cell functions (Gill 2001). Gene expression can be regulated at various levels, including transcription, messenger RNA (mRNA) splicing/ mRNA stability (post-transcription) and during/post-translation (Day and Tuite 1998). Transcriptional regulation, specifically post-transcription will be the focus in this project.

Transcription is the initial step in gene expression in which DNA is copied into a complementary strand of RNA, known as primary transcript (pre-mRNA) (Persson and Mueller 2015). The initiation of transcription can occur through a signal inside or outside the cell, which is important as the altering of transcription in response to distress within the extracellular environment is necessary for the host's response, particularly in a diseased state (Deutschman 2005). The start of transcription occurs with pre-initiation. This process requires a promoter region, which is found upstream to the site of transcription. During this process, regulators known as transcription factors can bind onto DNA resulting in activation or repression of transcription, called enhancers or repressors, respectively (Persson and Mueller 2015; Schöler, Wilmanns, and Reményi 2004). Another group of regulators are known as nuclear receptors, these transcriptional factors regulate gene expression in a ligand-dependant manner, by activating or repressing expression of target genes through direct binding to DNA response elements (Glass and Rosenfeld 2000).

Prior to the translation of proteins, post-transcriptional modification of pre-mRNA occurs. This includes the cleavage of large RNA precursors, 3' end polyadenylation and 5' end capping, methylation of the nucleotides within the mRNA and splicing of introns (Revel and Groner 1978). Post-transcription is regulated by various mechanisms. One of these mechanisms is alternative RNA splicing which involves the determination of which portion of the coding sequence within mRNA will be included in the final transcript. This results in several protein isoforms that differ in their peptide sequence and consequently chemical and biological activity (Black 2003). The enzymatic

deamination of Adenine to Inosine is another mechanism. This mechanism can result in the change of splicing patterns of RNA or a change in the meanings of codons i.e. a change in a C codon to a U codon can result in a stop codon, therefore truncating a large protein prematurely (Alberts 2002). Other mechanisms include proteins that are able to bind to the 5' untranslated region (UTR) of mRNA (Alberts 2002), for example poly(A)-binding protein (PABP). PABP binds to the 5'UTR of its mRNA leading to the autoregulation of PABP translation (Wu and Bag 1998). Additionally, small nucleotide sequences can bind to the 3' UTR of mRNA and mediate post-transcriptional repression (Friedman et al. 2009; Alberts 2002). The main mechanism of interest for this research is post-transcriptional regulation through the binding of small nucleotide sequences to the 3'UTR region of mRNA, in particular micro-RNAs (miRNA).

1.4.1. Micro RNAs

miRNAs are a family of endogenous, single stranded, non-coding RNAs that are 21-25 nucleotides in length (He and Hannon 2004; Obernosterer et al. 2006). Most miRNAs are conserved across species, which indicates evolutionary importance of miRNAs as essential regulators of biological pathways and processes (Christopher et al. 2016). Transcription of miRNA occurs in the cell nucleus, through the action of RNA polymerase II (Teruel-Montoya, Rosendaal, and Martinez 2015), following their transcription, these primary miRNAs are cleaved by RNase III to a hairpin-like precursor (pre-miRNA) (Gurianova et al. 2015). Once cleaved to pre-miRNA, they can be transported from the nucleus into the cytoplasm via the exportin 5 pathway for further processing by RNase III Dicer, generating a mature miRNA

(Teruel-Montoya, Rosendaal, and Martinez 2015; Gurianova et al. 2015). Mature single stranded miRNA assembles into RNA-induced silencing complexes (RISC) along with other associated proteins, this is then able to bind to the 3' UTR of target gene's mRNA and regulate its expression (Fig. 1.4.) (Teruel-Montoya, Rosendaal, and Martinez 2015; Maniataki and Mourelatos 2005).

While plant miRNAs usually interact with their target gene's mRNAs through perfect base pairing, a considerable number of animal miRNAs interact through imperfect base pairing (Carrington and Ambros 2003). Imperfect base pairing occurs as the 5'-end of miRNAs are more likely to have a higher number of bases that are complementary to the target gene's mRNA than the 3'-end does (Felekkis et al. 2010). The mature miRNA binds to the miRNA recognition elements (MRE) within the 3' UTR of its target gene's mRNA, through a region knows as the seed region, resulting in the inhibition of gene expression (Felekkis et al. 2010). It has been seen that within the seed region of miRNA, there are 7 nucleotides that match their target gene's mRNA, this can be seen in Figure 1.5. as nucleotides 2-8. (Grimson et al. 2007). While this 7-nucleotide seed region is sufficient for gene regulation it has been shown to be able to be disrupted by a single point mutation at the site of binding (Brennecke et al. 2005; Lai 2005).

The first miRNA that was found in humans was *let*-7. When incorrectly regulated, this miRNA leads cells into a less differentiated state, which results in the development of cell-based diseases such as cancer (Roush and Slack 2008). Since the discovery of *let*-7, 18,226 miRNAs have been described in animals, plants and viruses. This includes 1,921 that were found

to be encoded in humans (Kozomara and Griffiths-Jones 2011). The target sites of miRNAs have been a point of interest. In one study, Brennecke et al. (2005) presented evidence to demonstrate that a single miRNA can have approximately 100 target sites. This provided an indication towards miRNAs being regulators of numerous protein-coding genes. Correspondingly, one gene can be regulated by multiple and/or different miRNAs, indicating that the relationship between miRNAs and their targets is not a one-to-one association (Hashimoto, Akiyama, and Yuasa 2013).

Through extensive studies, it has been found that miRNAs act as key molecules in various physiological processes, including hematopoietic cell differentiation, regulation, apoptosis and development (Kim et al. 2004). They also have a role in the pathological processes of all human diseases (Di Leva and Croce 2013) for example, miR-155 in inflammatory disease, miR-122 in hepatitis C virus (HCV) mediated disease, miR-21 in hepatocellular carcinoma (HCC) and miR-10b in glioblastoma, (Christopher et al. 2016). While miR-122 is a key factor in liver development, it also plays a role in the life cycle of certain liver pathogens, specifically HCV. By binding to the 5' UTR of the HCV genome, it enhances the translation and replication of the virus (Christopher et al. 2016; Bandiera et al. 2015). Additionally, overexpression of miR-21 was seen in HCC tumour cells, with a decrease in tumour suppressor genes and miR-10b overexpression was seen to be related to the initiation of cell migration and invasion (Christopher et al. 2016). Similarly, studies have shown that overexpression of miR-155 results in the proliferative response of T-helper cells which induces chronic inflammation (Christopher et al. 2016; O'Connell et al. 2010). It is evident

that the dysregulation of miRNAs contributes to the pathogenesis and progression of most human malignancies (Nedaeinia et al. 2016)

In addition to human diseases, miRNAs have been demonstrated to have a role in the regulation of haemostasis and coagulation. For example, they have been reported as regulators of haemostatic proteins during developmental haemostasis (Teruel et.al. 2011). Alternatively, in adults the miRNA regulation of gene expression can produce an effect in which varying intensities of haemostatic proteins may be the potential cause of bleeding or clotting disorders (Teruel et al. 2011). Regulation of gene expression has also been seen within platelets. Through genome-wide profiling, Nagalla et al. (2011) found that there are 284 miRNAs expressed within platelets. With additional studies demonstrating that these miRNAs have a role in both normal and diseased human megakaryocytopoiesis and in the function of platelets (Garzon et al. 2006; McManus and Freedman 2015).

Supplementary to their key role in haemostasis and cellular processes, miRNAs have also been shown to have great therapeutic potential (Baumann and Winkler 2014). Findings have suggested that there are certain miRNAs that can act as tumour suppressors or oncogenes, with studies showing that the cancer phenotype of some cells, when treated with miRNA mimics or inhibitors, can be reverted to normal (Yeung and Jeang 2011). There have been cases of both artificial introduction and inhibition of miRNA in disease models with Kumar et al. (2008) describing the first use of artificially introducing miRNA as a tumour suppressor *in vivo*.

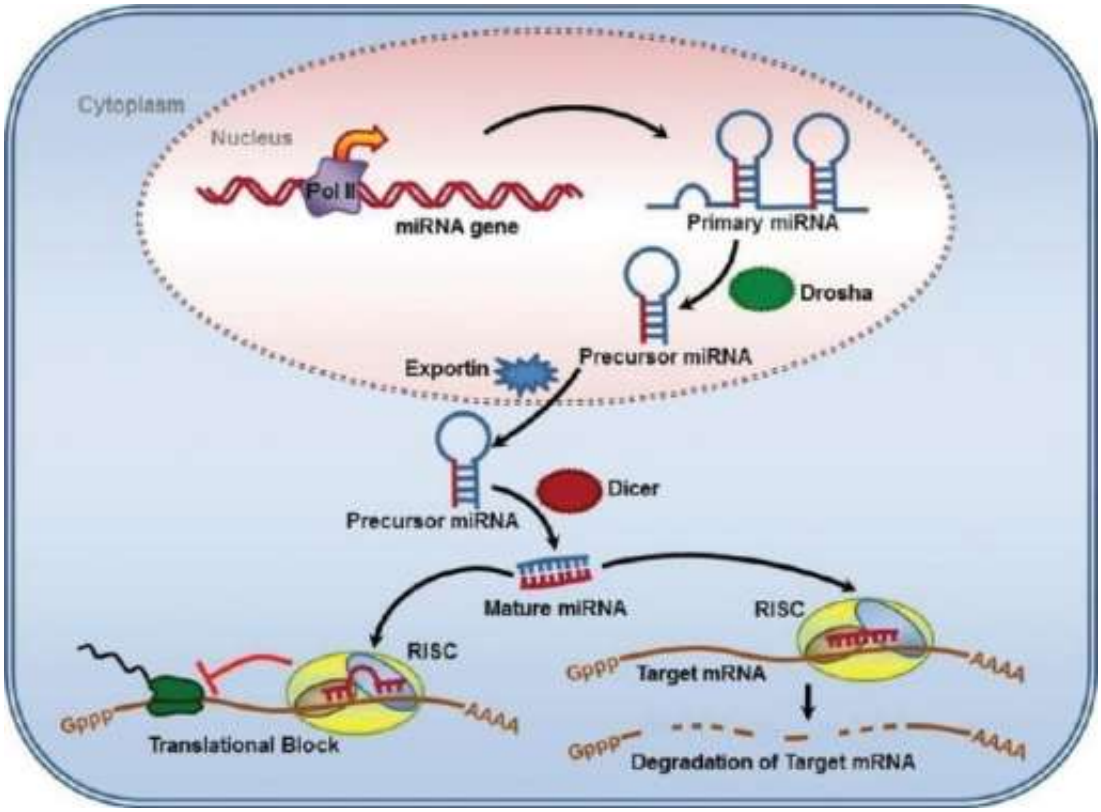


Figure 1.4. Schematic representation of miRNA biogenesis and action. Transcription of miRNA in the nucleus of cells by the action of RNA polymerase II (pri-miRNA). RNase III then cleaves Pri-miRNA to pre-miRNA. Pre-miRNA is exported out of the nucleus to the cytoplasm by Exportin 5 where it is subsequently processed by RNase III dicer to generate mature miRNA. One strand combines with RISC, which enables it to recognise target mRNA resulting in inhibition of gene expression.

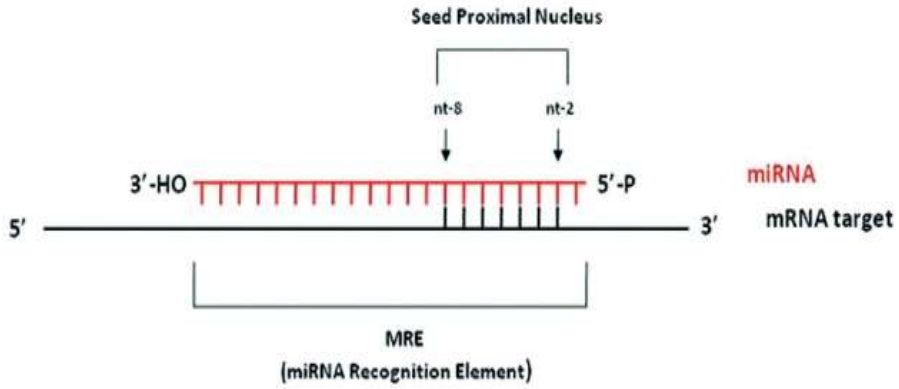


Figure 1.5. Imperfect base pairing of miRNA to its target mRNA. Mature miRNA base pairs bind to the MRE elements within the 3' UTR of target mRNA. The binding occurs in the 'seed region' which includes nucleotides 2-8 from the 5' end of the miRNA (Felekkis et al. 2010).

1.4.2. miR-494

There are 54 miRNAs found within the genomic region of chromosome 14q32.31, all of which have been seen to be either downregulated or overexpressed in certain cancers. They have therefore been identified as having tumour suppressive or oncomiR qualities. One of these miRNAs is miR-494 (Shahar et al. 2016; Liu et al. 2015). The downregulation of miR-494 has been reported in studies related to breast cancer, gastric carcinoma, oral cancer and pancreatic cancer, among others (Zhao, Liang, and Fu 2016; Ma et al. 2015; Liborio-Kimura, Jung, and Chan 2015). While various other studies have reported miR-494 as being overexpressed in numerous different cancers, such as acute myeloid leukaemia, lung cancer, HCC and colorectal cancer (Diakos et al. 2010; Ohdaira et al. 2012; Liu et al. 2015; Sun et al. 2014), with evidence demonstrating that the key function of miR-

494 in these cancers is proliferation, migration and invasion through the targeting of specific genes (Liu et al. 2015).

Supplementary studies have looked at miR-494 in association with other diseases and cellular processes. Wang et al. (2010) saw that miR-494 was downregulated in ischemia/ reperfusion-induced cardiac injury, while Welten et al. (2014) saw that miR-494 was a regulator of most of the target genes involved in neovascularisation. It was demonstrated that the in vivo inhibition of miR-494 resulted in the upregulation of these target genes and consequently an increase in neovascularisation after ischemia. Similarly, Yamamoto et al. (2012) reported miR-494 as a regulator of mitochondrial biogenesis in skeletal muscle, and when inhibited an increase in the mitochondrial levels was seen. Tay et al. (2013) investigated the way in which miR-494 regulates the expression of *PROS1* and consequently the expression of PS. It was seen that miR-494 expression was elevated in the presence of oestrogen which resulted in increased binding to sites found on the *PROS1* gene, subsequently leading to reduced PS levels.

The binding of miR-494 to sites found on the *PROS1* gene is of particular interest in this project. Using three binding site prediction programs (TARGETSCAN (http://www.targetscan.org), MIRANDA (http://www.microrna.org) and REGINA (http://regrna.mbc. nctu.edu.tw)) Tay et al. (2013) identified putative bindings sites for miR-494 within the 3' UTR of *PROS1* (Fig. 1.6.). It was found that there are three locations in which miR-494 is predicted to bind. Through computational analysis, site 1 was classified as an 8-mer binding site, which demonstrates 8 nucleotides as having complementary binding within the seed region, and sites 2 and 3

were classified as a 7-mer binding site, with nucleotides 2-8 having complementary binding within the seed region. These locations were subsequently ranked in order of binding strength with miR-494, using a prediction score that was found through the binding site prediction programs (Table 1.2). It was noted that a more negative score correlated to a more efficient site, therefore ranking the binding sites found on the *PROS1* 3' UTR as 1 being the strongest, followed by 3 and finally 2 (Tay et al. 2013).

While there are numerous studies relating to the therapeutic potential of miR-494 regulation in various diseases, the decreased expression of PS through miR-494 binding to the *PROS1* 3' UTR is a novel mechanism that has only been looked at in one study by Tay et al. (2013). The study demonstrated that miR-494 downregulates the expression of the *PROS1* gene, which resulted in the downregulation of PS (Tay et al. 2013). It is evident that miR-494 has various roles through the targeting of numerous genes including cell cycle regulators, regulators of DNA replication and transcription factors (Tay et al. 2016). A consequence of having a high number of target genes is that any change in miR-494 expression and availability can have a major impact, it's complete inhibition can therefore lead to physiological processes stemming from different causes (Welten et al. 2014). The ability to inhibit miR-494 binding to the PROS1 gene is a new concept. Accordingly, it is

proposed that the binding of miR-494 to *PROS1* mRNA will be the target for inhibition in this project.

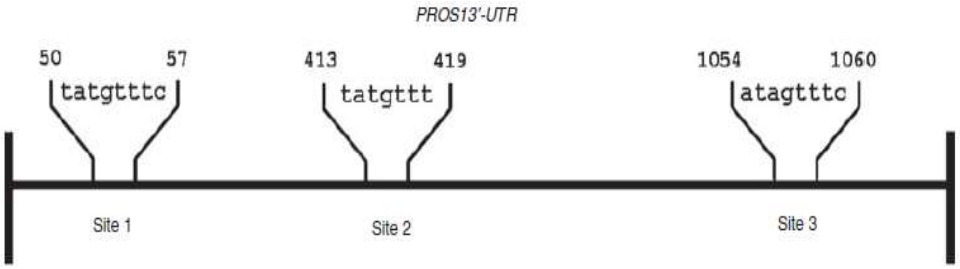


Figure 1.6. Computational analysis of *PROS1* 3'UTR, to identify binding sites for miR-494. Three sites were found at nucleotides 50-57, 423-419 and 1054-1060 (Tay et al. 2013).

Table 1.2. Binding site prediction scores using computational analysis

Nucleotide (nt) position of binding site	Target scan context score	miRanda miRSVR Score	RegRNA minimum free energy
1 – nt 50-57	–0.27	–1.25	–14.20
2 – nt 413-419	-	-	–8.80
3 – nt 1054-1060	–0.14	–0.78	–11.90

(Tay et al. 2013)

1.5: Principles of Lipid Transfection (Lipofection)

To analyse the effects of miRNAs on their target gene mRNA, studies have used the method of transfection using their selected cell line. Eukaryotic cells are capable of taking up exogenous DNA, with a portion of this DNA being taken into the nucleus to express either transient or stable genes (Felgner et al. 1987). The transfer of genes *in vivo* has been seen through the use of cationic lipids, with promising results for therapeutic use *in vivo* (Zabner 1997). The mechanism of action by which a cationic lipid takes up DNA for transportation into a cell depends of the lipid structure which is formed by a

hydrophobic anchor domain, a hydrophilic cationic headgroup and a linker (Fig. 1.7.).

The hydrophobic anchor is a nonpolar hydrocarbon moiety consisting of single and double chained hydrocarbons and cholesterol. The single chained hydrocarbons are important as they form micelles in solution, while the double chained hydrocarbons are able to form liposomes and the cholesterol helps to stabilise the liposomes. The linker is important in establishing an ideal network between the head group of the cationic lipid and the negatively charged phosphates of DNA (Chesnoy and Huang 2000) and is usually an ether or ester (Niculescu-Duvaz, Heyes, and Springer 2003).

The cell membrane is negatively charged, therefore the mechanism by which a lipid-DNA complex interacts and enters the cell is dependent on the overall charge of the vesicle being positive. The negatively charged DNA contained within the lipid must then be released to alter expression within the cell's nucleus. It first enters the cytoplasm which has been seen to occur through possible disruption of the endosomal membrane which occurs through an interaction between the cationic lipids and anionic molecules that are present within the membrane. Once the DNA is within the cell's cytoplasm it enters the nucleus via free diffusion (<70 kDa) or active transport (>70 kDa) where it can alter expression (Fig. 1.8.) (Chesnoy and Huang 2000).

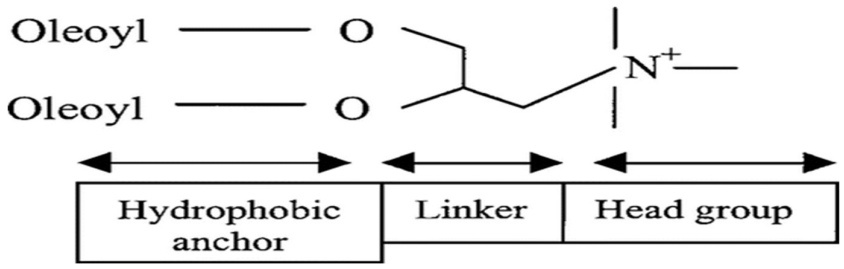


Figure 1.7. Graphic example of the three important groups of a cationic lipid: The hydrophobic anchor, the linker and the head group. The hydrophobic anchor is responsible for liposome formation and the linker ensures optimal contact between the head group and negatively charged DNA (Chesnoy and Huang 2000).

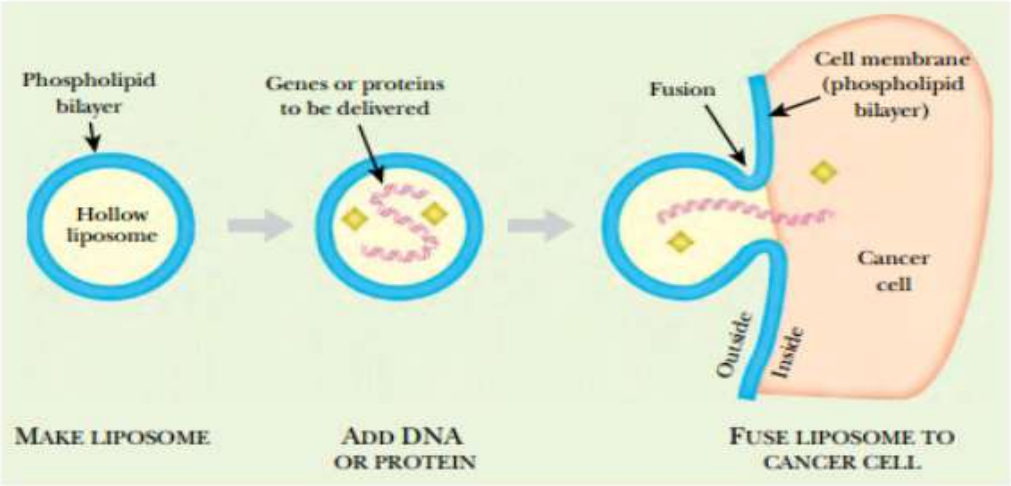


Figure 1.8. Representation of transfection using a lipofection method. A hollow liposome takes up desired DNA/ RNA which is then added to a chosen cell line where a fusion process occurs during an incubation period.

1.6: micro-RNA Therapeutics

The therapeutic potential of miRNAs has been greatly documented in studies relating to cancer and other diseases. Using miRNA gene-knockout studies, there has been a propagation of insight into the functions of miRNAs. Ventura et al. (2008) showed the significance of the miRs-17-to-92 family of clusters using gene knockout in a mouse model. The absence of miRs-17-to-92 led to the increase of a pro-apoptotic protein and a decrease of B cell development, this resulted in postnatal death in 100% of cases. Additionally, a study by Poy et al. (2009) showed an alternate gene knockout in a mouse model. Mice lacking miR-375 exhibited an increase in pancreatic α-cell numbers leading to an increase in gluconeogenesis and glucose output while there was a decrease in pancreatic β-cell mass due to impaired proliferation. There have been many different miRNA gene knockout studies with diverse results. While most have been associated with a loss of function phenotype, there are a small amount that result in no obvious phenotype (Liu et al. 2008; van Rooij et al. 2009; Williams et al. 2009).

An alternative approach to miRNA gene knockout is the use of synthetic inhibitors. There are various forms of inhibitors, including: Antisense oligonucleotides (ASO), locked nucleic acids (LNA)-antimiRs, antagomirs and aptamers. ASOs are designed to be the reverse complement of the target site of a mature miRNA, while being chemically modified to ensure resistance to degradation and enhance affinity of binding. Through Watson-Crick base pair hybridisation, ASOs will bind to their target mRNA, hindering the ability of miRNA interaction and repression of target mRNAs (Fig. 1.9.). LNA-antimiRs are partially modified, 8 nucleotide ASOs that are designed to

target the 5' seed regions of miRNAs (Ling, Fabbri, and Calin 2013). Like ASOs, the mechanism of action of LNA-antimiRs involves Watson-Crick base pair hybridisation, however these molecules bind to the miRNA itself, hindering its ability to bind to its target mRNA (Kearney et al. 2008). In 2005, Rajeev et al. (2005) designed a novel class of chemically engineered oligonucleotides, termed 'antagomirs'. Developed as a pharmacological approach for silencing miRNAs *in vivo*, these single stranded RNA molecules are cholesterol-conjugated and are designed to be complementary to the mature target miRNA (Mattes, Yang, and Foster 2007). Aptamers have been used for the delivery of oligonucleotides *in vivo*. Subramanian et al. (2015) observed that with the use of an aptamer, targeting the precursor form of miR17~92 cluster resulted in downregulation of the mature miRNAs encoded by the cluster while their cognate mRNA targets were upregulated. The downregulation of miRNAs encoded by this cluster induced apoptosis and inhibited cellular proliferation in retinoblastoma cell lines.

An alternative to oligonucleotides are miRNA inhibitors that are expressed as RNAs from transgenes, within cells (Neilson, Sharp, and Ebert 2007). These are termed 'miRNA sponges' and are RNA transcripts that contain multiple binding sites that associate with high affinity to specific miRNAs, preventing their interaction with target gene mRNAs (Barta, Peskova, and Hampl 2016). Vectors that encode these miRNA sponges are transiently transfected into cells. This allows the miRNA sponges to act as a decoy target, resulting in the decreased availability of these miRNAs to bind onto target gene mRNA (Neilson, Sharp, and Ebert 2007). In addition to synthetic inhibitors and miRNA sponges, researchers have also considered the use of small-

molecular drugs that target specific miRNAs. Bose et al. (2012) showed the efficient repression of miR-21 levels with the use of streptomycin. The mechanism of action for this drug involves the direct binding to the precursor form of miR-21, causing interference with the downstream processing by Dicer. Streptomycin was seen to be specific to miR-21 as it failed to repress other related miRNAs that were tested. Similarly, Murata et al. (2013) looked at the interaction between xanthone and thioxanthone derivatives and miR-29a. It was found that an aminoalkoxy-substituted thioxanthone derivative known as X2SS, was a potent inhibitor of the preliminary miR-29a Dicer reaction. Other small-molecular drugs that have been investigated, include peptides and amino-glycosides (Maiti, Nauwelaerts, and Herdewijn 2012). The generation of RNA binding macrocyclic helix-threading peptides through solid-phase ring closing metathesis have been shown to be able to bind to naturally occurring precursor miRNAs (Krishnamurthy et al. 2007). Similarly, it was seen that some aminoglycosides can bind to precursor miRNAs, but are ineffective in the inhibition of miRNA maturation. Regardless, the binding to precursor miRNAs creates the potential to interfere with other steps within the miRNA pathway, providing a possible role of the side effects of this class of antibiotic (Maiti, Nauwelaerts, and Herdewijn 2012). These examples have all provided favourable leads to further develop potent small molecular miRNA-antagonist drugs.

As a contrast to diseases with overexpressed miRNAs, there are also conditions in which miRNAs are downregulated (Shimono et al. 2009; Kumarswamy et al. 2012). This is a common scenario in cancers, with several downregulated miRNAs having tumour suppressive qualities,

providing a need for them to be restored to their normal levels. Restoration of miRNA levels can be achieved through the delivery of three different forms of miRNA therapy: a synthetic miRNA oligonucleotide mimic, small molecules used to reverse the inhibition of miRNA or the miRNA transgene using a DNA vector (Henry, Azevedo-Pouly, and Schmittgen 2011). Studies have considered the possible use of miRNA mimics as therapeutic agents with the potential to be tumour and growth suppressors. Long et al. (2009) reported the first case in which miR-let-7a is downregulated in human laryngeal squamous cancer, thus proposing that delivery of a synthetic mimic could inhibit cell growth and induce cell apoptosis. Reid et al. (2013) demonstrated the consistent downregulation of miR15/16 in a malignant pleural mesothelioma cell line. Through this study, the researchers demonstrated that the introduction of a miR-16 mimic resulted in tumour regression *in vitro*, which provides promising results for future therapeutic agents. In 2013, the first synthetic miRNA mimic was used in a clinical setting (Bouchie 2013). MRX34 is a mimic of miR-34a, a miRNA that is downregulated in primary liver cancer resulting in the suppression of miR-34-mediated apoptosis (Hermeking 2010). The therapeutic MRX34, a double-stranded RNA, is transported to the liver using liposomal nanoparticles to increase delivery into the cell (Schmidt 2014). This therapeutic mimic is known to directly regulate 24 oncogenes involved in cellular process such as the cell cycle, proliferation and anti-apoptosis.

There are several mechanisms which account for the downregulation of miRNAs in cancers, these include the failure of miRNA post-transcriptional regulation, transcriptional repression by oncogenic factors, a mutation of the

TARBP2 miRNA processing gene that expresses the TAR RNA-binding protein 2 (TRBP) and the down regulation of the DICER1 miRNA gene (Melo et al. 2011). These mechanisms have been the basis for the development of small molecules to reverse the inhibition of miRNAs. The use of Enoxacin, a synthetic antibacterial compound, has been seen to promote the interaction between TRBP and RNAs, which enhances the small interfering RNA (siRNA)- mediated mRNA degradation, therefore promoting the biogenesis of endogenous miRNAs (Szulwach et al. 2008). Another molecule that has been used to aid the upregulation of miRNA expression was a quinazoline based compound. This use of this compound resulted in the global upregulation of miRNAs, with selective enhancement of miRNAs with tumour suppressive qualities, this means they can cause the death of cancer cells through apoptosis. Three quinazoline compounds were found, each with a core 2,4-diphenyl-quinazoline component that is seen as a promising platform and as a tool in the designing of more effective activators of miRNA expression (Nahar et al. 2014).

Restoring miRNA levels in certain diseases via the delivery of a miRNA transgene using a vector is seen in the study by Brown et al. (2007). Through the tagging of the transgene hF.IX with miR-142-3p, they were able to produce long term delivery using a lentiviral vector in immunocompetent haemophilia B mice. Through this method, it was seen that the phenotype of the mice improved. Further evaluation of this vector system and transgene is still required, but there are encouraging results that could lead to new therapeutic treatments in haemophilia B, and other genetic diseases.

It is seen that miRNAs have emerged as potential therapeutic agents in various diseases, but problems in transferring this to a clinical setting have arisen due to the lack of an effective delivery system to diseased sites or the potent inhibition of overexpressed miRNAs without degradation. Through the implementation of non-modified synthetic oligonucleotides, like ASOs, it was quickly discovered that they are degraded rapidly by endogenous exonucleases and endonucleases present within cells and serum. Depending on their mechanism of action and target nucleic acids, different chemical modification and delivery approaches have been employed (Lennox and Behlke 2011). The altering of delivery method for phosphodiester oligonucleotides to improve stability *in vivo* was looked at by De Oliveira et al. (2000). Anionic lipids were used for the delivery of these oligonucleotides. Even though it is seen that cationic lipids increase resistance of degradation by nucleases, they are limited by their cellular toxicity, therefore not suitable for *in vivo* delivery. The use of DOPC/OA/CHOL and DOPE/OA/CHOL pH sensitive anionic lipids were looked at to determine the most efficient formulation. It was seen that the DOPC/OA/CHOL liposome had a higher stability, therefore providing greater protection of the oligonucleotides. Phosphodiester oligonucleotides are relatively easy to synthesise, however once in the nucleus, rapid degradation occurs via intracellular enzymes. This produces degradation products that may be cytotoxic and exert anti-proliferative effects (Dias and Stein 2002).

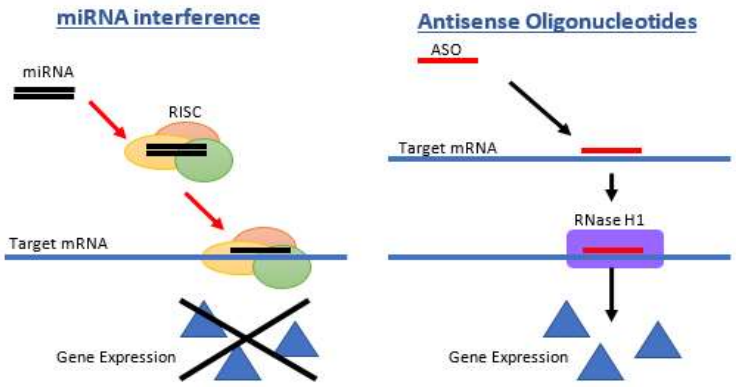


Figure 1.9. Schematic representation of miRNA binding to its target mRNA and repressing gene expression. Compared to an antisense oligonucleotide binding and allowing RNase H1 to catalyses the cleavage of RNA and gene expression as normal.

1.6.1. Chemical Modifications

Chemical modifications that have been carried out to ensure the strengthening of ASOs include a phosphorothioate modification (Fig 1.10) that substitutes a sulphur atom for a non-bridging oxygen within the phosphate backbone, resulting in the reduction of degradation by endogenous nucleases. These modifications are able to be selectively placed throughout an oligonucleotide sequence, however this change results in a reduced binding affinity and a lowered melting temperature (T_m) (Lennox and Behlke 2011).

Additional 2'-O-methyl RNA (2'OMe) modifications can be applied to a phosphorothioate backbone. The use of these modified ASOs have advantages over unmodified phosphodiester and unchanged phosphorothioate ASOs. Advantages occur as they have a much higher T_m and consequently binding affinity, they also interfere with nuclease

interaction with and degradation of single stranded ASOs in comparison to DNA. Despite their advantages, 2'OMe ASOs are not entirely nuclease resistant, therefore alternate modifications are needed to ensure they aren't rapidly degraded (Lennox and Behlke 2011). Rajeev et al. (2005) modified 2'OMe ASOs with multiple phosphorothioate bonds between internucleotide linkages found at the end of the molecule. This was done to prevent exonuclease attack of the molecule and subsequent degradation. Additionally, a cholesterol group was attached the 3'end of the molecule for more efficient delivery *in vivo*. ASOs with complete phosphorothioate modifications were also tested, but due to the reduced binding affinity and lowered melting temperature, no reduction in miRNA levels occurred.

To enhance the T_m of ASOs, LNA modifications along with the phosphorothioate modified backbone are used. Compared to 2'OMe ASOs, LNA modifications have been seen to be very potent (2'OMe - ~80mg/kg, LNA – 5-25 mg/kg in rodents) (Lennox et al. 2013). The LNA modification is usually placed within a 2'OMe backbone at every third position to reduce the possibility of hairpin loops and self-dimer hybridisation that occurs with high affinity binding modifications of ASOs (Lennox et al. 2013). While LNA modified ASOs are seen to be useful *in vitro*, adverse outcomes can occur *in vivo* due to off-target effects that are caused by non-specific binding to non-targeted molecules and miRNAs. It has also been seen that the use of as few as four LNA ASOs can result in severe hepatotoxicity *in vivo* (Lennox et al. 2013)

One of the major problems with the use of ASOs is the target sequence. When using ASOs that contain DNA, they are able to form RNA-DNA hybrids

with act as a substrate for RNase H1, promoting the cleavage of the mRNA target. In contrast, an oligonucleotide termed ‘morpholino’ has been seen to form RNA-morpholino hybrids that do not act as a substrate for RNase H1, therefore not resulting in the degradation of the target mRNA. Morpholino oligonucleotides have altered backbone linkages when compared to phosphodiester and phosphorothioate backbones (Fig. 1.10. A). These oligonucleotides bind by Watson-Crick base pairing as do ASOs, but because of the modified backbone, morpholinos are resistant to digestion by nucleases. The backbone also lacks a negative charge which has been thought to make morpholinos less likely to interact non-selectively with intracellular proteins (Corey and Abrams 2001). In comparison to the targeting success rate of phosphodiester oligonucleotides, being 10-20%, morpholinos have been shown to be in the range of 70 – 80%, due to their altered structure having a non-ionic subunit making them resistant to degradation. Additionally, morpholinos have not exhibited any toxic effects *in vivo* (Summerton 2005)

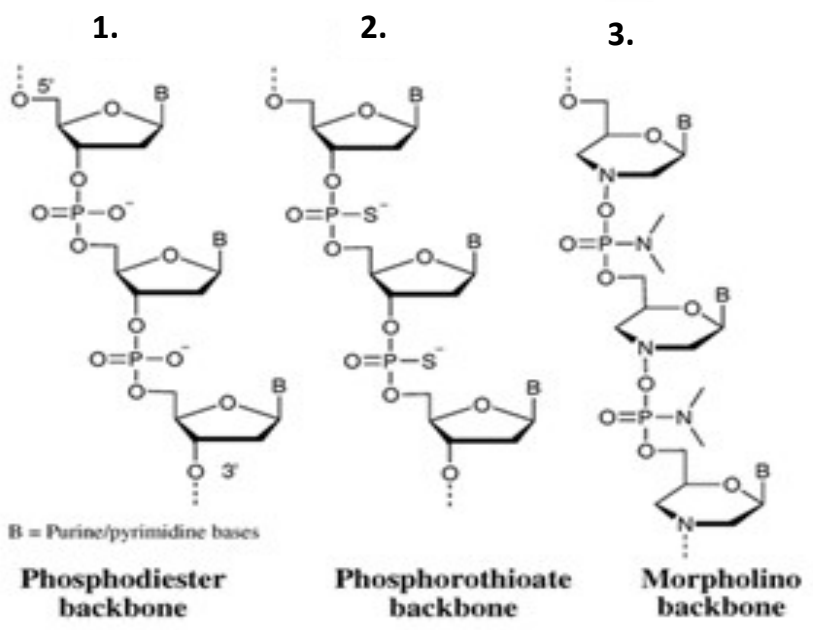


Figure 1.10. Structures of natural and modified oligonucleotide backbones: (1) natural phosphodiester; (2) phosphorothioate; (3) morpholino (Singh, Murat, and Defrancq 2010) .

1.7: Statement of Aims

The potential treatment of cancers and other diseases has been seen in various studies through the use of oligonucleotides and their chemically modified equivalents. There are still limitations that need to be overcome with the use of these molecules, however, there have been favourable results that have provided insights into the functions of miRNA regulation in these diseases. The main hypothesis of this research is therefore the introduction of 2'OMe phosphorothioate or LNA modified oligonucleotides against the three miR-494 binding sites found within *PROS1* mRNA. This will block the mRNA and miR interaction and consequently lead to increased PS expression.

The aims for this research are therefore as stated:

1. To evaluate specifically targeted 2' OMe or LNA oligonucleotides designed to block the miR-494/*PROS1* interactions, increasing the *PROS1* mRNA transcript lifespan leading to an overall increased expression of PS.
2. To determine the most efficient combination of specifically targeted 2'OMe or LNA oligonucleotides that will result in the highest level of *PROS1* mRNA transcripts and ultimately PS

Chapter 2: Materials

2.1. Chemical Reagents

2.1.1. Cell culture

Item	Company
HuH-7 cell line	-
DMEM (1x)	Gibco® by Life Technologies, USA
Phosphate Buffered Saline (1x) (PBS)	Gibco® by Life Technologies, USA
TrypLE™	Gibco® by Life Technologies, USA
Foetal Bovine Serum	PAA Laboratories GmbH, Germany
Penicillin Streptomycin	Gibco® by Life Technologies, USA
Sodium Pyruvate	Gibco® by Life Technologies, USA
Non-essential Amino Acids	Gibco® by Life Technologies, USA
Metafectene® Pro	Biontex Laboratories, Germany
Lipofectamine™ 3000 Reagent	Invitrogen, Australia
Opti-MEM™	Gibco® by Life Technologies, USA
Pre-miR-494	Ambion®, Australia
Pre-miR-NC	Ambion®, Australia
2'O'Methyl Phosphorothioate Oligonucleotides	Supplied by Rakesh Veedu, CCG, Murdoch
Locked Nucleic Acid (LNA) Oligonucleotides	Supplied by Rakesh Veedu, CCG, Murdoch

2.1.2. Bacteria Culture and pRR5-Duo Plasmid Extraction

Item	Company
Ampicillin Sodium Salt	Sigma-Aldrich Co., USA
Yeast Extract	Amresco® LLC, USA
Tryptone	Amresco® LLC, USA
Sodium Chloride	Amresco® LLC, USA
Sodium Hydroxide	Sigma-Aldrich Co., USA
NucleoBond® Xtra plasmid purification Midiprep kit	Machery-Nagel GmbH & Co. KG, Germany
NucleoBond® Xtra plasmid purification Miniprep kit	Machery-Nagel GmbH & Co. KG, Germany

2.1.3. Luciferase Assay

Item	Company
5x Lysis Buffer	Gene Stream Pty. Ltd, Australia
Firefly Glow Assay Buffer	Gene Stream Pty. Ltd, Australia
50x Firefly Enhancer	Gene Stream Pty. Ltd, Australia
10x Luciferin	Gene Stream Pty. Ltd, Australia
Flash and Glow Gaussia Assay Buffer	Gene Stream Pty. Ltd, Australia
100x Coelenterazine	Gene Stream Pty. Ltd, Australia

2.1.4. Total RNA extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Item	Company
TRIzol™ Reagent	Invitrogen™ by Life Technologies, USA
10mM dNTP	Invitrogen™ by Life Technologies, USA
50µm Random Hexamers	Invitrogen™ by Life Technologies, USA
5x First Strand Buffer	Invitrogen™ by Life Technologies, USA
0.1M Dithiothreitol (DTT)	Invitrogen™ by Life Technologies, USA

RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen™ by Life Technologies, USA
SuperScript® III Reverse Transcriptase Enzyme	Invitrogen™ by Life Technologies, USA

2.1.6. Quantitative Polymerase Chain Reaction (qPCR)

Item	Company
TaqMan® Universal Master Mix II, no UNG	Applied Biosystems™ by Life Technologies
TaqMan® Gene Expression Assay, ACTB (human	Applied Biosystems™ by Life Technologies
TaqMan® Gene Expression Assay, GAPDH (human)	Applied Biosystems™ by Life Technologies
TaqMan® Gene Expression Assay, *PROS1* (human)	Applied Biosystems™ by Life Technologies

2.2. Laboratory Equipment

2.2.1. General

Item	Company
Falcon™ 5mL tube	Thermo Fisher Scientific, USA
Falcon™ 50mL tube	Thermo Fisher Scientific, USA
1.5ml Eppendorf tubes	Sarstedt, Nümbrecht, Germany
Single channel pipettes	Axygen®, Corning Inc, USA
Pipette Controller - MotoPet®	Axygen®, Corning Inc, USA
Nikon® H55OS	Nikon Instruments Inc., USA
Nanodrop 1000 Spectrophotometer	Nanodrop® Thermo Scientific, USA

2.2.2. Cell Culture, RNA Extraction and Transfections

Item	Company
Falcon™ Tissue Culture Flask, 75cm^2	Thermo Fisher Scientific, USA
Eve™ Cell Counting Slide	NanoEnTek Inc., USA
Countess™ Automated cell counter	Invitrogen™ by Life Technologies, USA
Falcon™ 24 well Tissue Culture Plate	Becton Dickinson Labware, USA

2.2.3. RT-PCR and qPCR

Item	Company
Microfuge® 16 Centrifuge	Centrifuge Beckman Coulter Inc., USA
CFX96™ Real-Time PCR Detection System	Bio-Rad Laboratories Inc., USA
CFX384™ Real-Time PCR Detection System	Bio-Rad Laboratories Inc., USA
C100™ Thermal Cycler	Bio-Rad Laboratories Inc., USA

2.2.4. Luciferase Assay

Item	Company
24 well cell tissue culture plate	Becton Dickinson Labware, USA
Micro assay 96 optical well plate	Greiner Bio-One, Germany
VictorTM Light Luminescence Counter	Perkin Elmer Inc., USA

2.2.5. Statistical Analysis

Programme	Company
Microsoft Excel	Microsoft® Corporation, USA.
Bio-Rad CFX Manager 3.0	Bio-Rad Laboratories Inc., USA

Chapter 3: Methods

3.1. Cell culture

3.1.1. Maintenance of the HuH-7 cell line

The human hepatocarcinoma cell line, HuH-7, was cultured in phenol red-free Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 10mM sodium pyruvate (NaPyr/$C_3H_3NaO_3$), 100U/ml $^{-1}$ penicillin, 10 µg/mL $^{-1}$ streptomycin and 10mM non-essential amino acids (NEAA).

The cell line was incubated and grown in 37˚C/5% CO_2 and monitored every 1-2 days using a Nikon Eclipse TS100 light microscope to observe cell confluency. The culture medium was replaced every 2-3 days and cells were passaged when they reached at 80-90% confluency. Cells were passaged by removal of culture medium followed by washing of the flask with 10mL of Phosphate Buffered Saline (PBS) to ensure the removal of any remaining culture medium. 5mL of 0.25% trypsin was added to the flask which was then placed in the 37ºC/5% CO_2 incubator for 2-3 minutes to enable cells to be detached from the flask surface. 5 mL of fresh culture medium was added to the trypsinised flask to inactivate the trypsin. The cells were then passaged at either 1:2, 1:5 or 1:10 dilutions into new flasks.

3.2. pRR5-Duo-miR-494-BAIT Vector

3.2.1. Vector backbone preparation

The digestion reaction was run using a thermocycler for 4 hours at 37ºC with 1ul/ul of pRR5-Duo-37 vector, 10X CutSmart buffer, 1µl Mlu1-HF, 1µl PmeI and 3µl dH_2O. The digested vector was then put into the thermocycler for 1hour at 37ºC and 15 minutes at 4ºC with Antarctic phosphatase treatment.

This included 10µl vector digest, 10X Antarctic phosphatase buffer, 1µl Antarctic phosphatase and 7 µl dH_2O. This digestion reaction was then put through a NucleoSpin® Gel and PCR Clean-up. Following the supplied protocol, the reaction mixture was made up to 100µl with H_2O and 200µl of buffer NTI was then added. A NucleoSpin® Gel and PCR Clean-up Column was then placed into a collection tube and the sample was loaded in. This was then centrifuged at 11000 x g for 30 seconds and the flow through was discarded. The silica membrane of the NucleoSpin® Gel and PCR Clean-up Column was then washed by the addition of 700µl of buffer NT3 which was then centrifuged at 11000 x g for 30 seconds. The flow through was once again discarded and this wash step was repeated. The silica membrane was dried by centrifuging at 11000 x g for 1 minute and DNA was eluted into a new 1.5ml Eppendorf tube through the addition of 30µl of Buffer NE to the NucleoSpin® Gel and PCR Clean-up Column. This was then incubated at 20-25ºC for 1 minute before being centrifuged at 11000 x g for 1 minute. The eluted PCR clean-up product was stored at -20ºC until needed.

3.2.2. Ligation of insert

The bait sequence oligonucleotides were designed as follows Mlu1—miR-494-pRR5-BAIT—PmeI (5'AAACGAGGTTTCCCGTGTATGTTTCAA3' and 5'CGCGTTGAAACATACACGGGAAACCTCGTTT3'). The oligonucleotide stocks were at 100µM each. They were diluted to 10µM, 1µM and 0.1µM through consecutive 1:10 dilutions with dH_2O. Respective concentrations of both sense and antisense oligonucleotides were added to separate tubes and placed on an 100ºC hot block for 15 minutes, then left to cool to 20-25ºC, forming the insert.

To run the ligase reaction, 1µl of each insert and 1 ng/µl digested vector were mixed with 10X ligase buffer, 1µl T4 Polynucleotide kinase and 6 µl dH_2O. This reaction was run for 12 hours at 16ºC using a thermocycler.

3.2.3. Transformation into chemo competent cells

Competent *Escherichia coli* (*E. coli)* were stored in -80ºC, they were then removed and thawed on ice for 20 minutes. 50µl of competent bacteria were mixed with 2µl of each ligation reaction in a 1.5µl Eppendorf tube, this was then incubated of ice for 30 minutes. 50 µl of competent bacteria was also mixed with 1 ng/µl pRR5-Duo vector which contained the *PROS1* 3' UTR. This was done as a control. The cells then underwent heat shock by being placed into a 42ºC water bath for exactly 45 seconds, then being placed back on ice immediately and incubated for 2 minutes. 250mL SOC media was added to each tube and then placed into a 37ºC shaking incubator for 1 hour. Following incubation, 150ul of each transformation was plated out on Lysogeny broth (LB) agar supplemented with 1µg/ml ampicillin. The plated-out bacteria were incubated over night at 37˚C.

The following day, plates were checked for colonies. Each separate colony was labelled, taken and grown separately in 5ml of pH 7.5 LB broth media for 12 hours in a 37˚C shaking incubator. The next day, NucleoSpin® Plasmid/ Plasmid (NoLid) Miniprep was performed on each colony. Following the supplied protocol, the cells were centrifuged for 11000 x g for 30 seconds and the supernatant was discarded. The pellet was resuspended in 250µl of buffer A1 and transferred to new 1.5ml Eppendorf tubes before the addition of 250µl of buffer A2. The tubes were inverted 8 times to mix before being incubated at 20-25ºC for 5 minutes. 300µl of buffer A3 was then added to

lyse the cells and the tubes were once again mixed by inversion until the samples turned from blue to colourless. The cell lysate was centrifuged at 11000 x g for 5 minutes. A NucleoSpin® Plasmid/Plasmid (NoLid) Column was then placed in a 2ml Collection Tube and the supernatant from the cell lysate was decanted into the column. This was centrifuged at 11000 x g for 1 minute and flow through was discarded. The silica membrane of the NucleoSpin® Plasmid/Plasmid (NoLid) Column was the washed using buffer 600µl of buffer A4 that was preheated to 50°C. This was then centrifuged at 11000 x g for 1 minute and the flow through was discarded. The silica membrane was dried by centrifuging at 11000 x g for 2 minutes and the collection tube was discarded. The DNA was eluted into a new 1.5ml Eppendorf tube by the addition of 50µl buffer AE, which was incubated at 20-25ºC for 1 minute followed by centrifuging at 11000 x g for 1 minute. The eluted DNA concentrations were measured using the Nanodrop® 1000 spectrophotometer. Samples were stored at -20°C until needed.

3.2.4. Sanger Sequencing

Presence of the miR-494-pRR5-BAIT insert was determined using the Big Dye Terminator reaction followed by Sanger Sequencing. The master mix was prepared with 2.5X sequencing buffer, 100ng of DNA was sequenced, 1µM P809 pRR5_miR_seq_FWD primer, 0.5µl/reaction of Big Dye Terminator and the volume made up to 20µl with dH_2O. The master mix was loaded into a 96 well half-skirt PCR reaction plate. The plate was placed in the Bio-Rad Real-Time PCR Detection System and cycled under the following conditions: 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes followed by 15ºC until removed.

The plate was then run through the PCR clean-up process using the Biomek FX^P Automated workstation, before being sequenced by the 3730XL DNA Analyser.

3.3. Transfection using dual luciferase reporter vector and miRNA precursors

3.3.1. Preparation of large scale dual luciferase reporter vector plasmid

E. coli glycerol stocks containing the pRR-5-Duo-37uo luciferase reporter vector (See Appendix, Section C) were used to inoculate 90mL of LB medium with 1µg/mL ampicillin. This was placed into a 37ºC shaking incubator, to grow for 12 hours. The following day, plasmid constructs required for transfection were extracted using the NucleoBond® Xtra plasmid purification Midi kit. Following the supplied protocol, cells were centrifuged at 6000 x g for 10 minutes at 4ºC. The supernatant was poured off and the pelleted cells were resuspended by pipetting up and down in resuspension buffer containing RNase A. 8mL of lysis buffer was then added to the cells and mixed by inverting the tube 5 times, this was then incubated at 20-25ºC for 5 minutes. The NucleoBond® Xtra column was equilibrated during the incubation time by the application of 15mL equilibration buffer, which was emptied via gravity flow. Following incubation, 8mL of neutralisation buffer was added to the lysate and immediately mixed by inversion of the tube. This neutralised lysate was then poured into the column filter and emptied via gravity flow. The column filter was then washed with 5mL of equilibration buffer wash, then discarded once the solution had emptied. The column itself was then washed with 8mL wash buffer. Elution of the plasmid DNA was performed using 5mL elution buffer to the column, and the flow-through

collected into a 15mL tube. 3.5mL 20-25ºC isopropanol was added to precipitate the eluted plasmid DNA before being centrifuged at 7000 x g and 4ºC for 30 minutes. The supernatant was removed, leaving the plasmid pellet, which was then washed with 2mL of 70% ethanol and centrifuged once more at 7000 x g for 5 minutes at 20-25ºC . The supernatant was once again removed and the pellet was air dried at 20-25ºC for approximately 15 minutes. The dried plasmid pellet was dissolved in 200µL of buffer TE which was then run through the Nanodrop 1000 spectrophotometer to determine its concentration. 50µl aliquots of extracted plasmid DNA were diluted to 1µg/µl and then stored at -20ºC until needed.

3.3.2. Transfection using luciferase reporter vectors and pre-miRNAs

HuH-7 cells were trypsinised (Section 3.1.1.) and the concentration and cell viability was measured using the Countess Automated Cell Counter. Cells were then seeded into 24 well plates at a density of $2.0x10^5$ cells/well and $1.5x10^5$ cells/well. The seeded plate was then incubated at 37˚C/5% CO_2 for 12 hours to allow cells to adhere before transfection. The following day, the seeded cells were checked for optimum confluency, being ~70-80%. The cell density with this optimum confluency was chosen for transfection. HuH-7 cells were co-transfected with 50nM of pre-miR-NC or pre-miR-494, 1µg/µl of pRR-5-Duo-REPORT luciferase construct containing the *PROS1* 3'UTR and 50 nM of 2'O-methyl phosphorothioate (2'OMe) or locked nucleic acid (LNA) oligonucleotide blockers.

3.3.3. Metafectene® Pro as lipofection reagent

The initial transfections were completed using the Metafectene® Pro lipofection reagent. This reaction mix was prepared by adding 2µl Metafectene® Pro and 48µl Opti-MEM™ Reduced Serum Medium (Phenol-red free) to the solution of 50µl Opti-MEM™, pre-miRNAs and luciferase construct with and without the 2'OMe oligonucleotide blockers. Once mixed by pipetting up and down once, the transfection mixture was incubated at 20-25ºC for 15 minutes before being added dropwise into the 24 well cell culture plate and incubated for 12 hours at 37ºC/ 5% CO_2.

3.3.4. Lipofectamine™ 3000 as lipofection reagent

Initially, Lipofectamine™ 3000 was used for comparative analysis against Metafectene® Pro. It was subsequently used for all further transfections. This reaction mix was prepared by adding 1.5 µl Lipofectamine™ 3000 to 24µl Opti-MEM™ to the solution of 25µl Opti-MEM™, pre-miRNAs and luciferase construct with and without the oligonucleotide blockers. Once mixed by pipetting up and down once, the transfection mixture was incubated at 20-25ºC for 15 minutes before being added dropwise into the 24 well cell culture plate and incubated for 12 hours at 37ºC/ 5% CO_2.

3.3.5. Luciferase Assay

The media in each well was removed following the transfection incubation period. 1x lysis buffer was then added to each well and allowed to incubate at 20-25ºC for 30 minutes. The luciferase assay was prepared using the RapidReporter® Firefly glow assay kit and RapidReporter® flash and glow assay kit from GeneStream. For the detection of Firefly activity, 60µl of Firefly glow assay buffer containing the 10x luciferin substrate was prepared

and incubated for 20 minutes at 20-25ºC, covered in foil to allow the substrate to reach a steady glow phase. For the detection of Gaussia activity, 60µl of flash and glow assay buffer containing the 100x coelenterazine substrate was prepared and incubated for 20 minutes at 20-25ºC, covered in foil to allow the substrate to reach a steady glow phase.

The lysate was then aliquoted into 20µl triplicates for both Firefly and Gaussia, in an optical 96-well reaction plate. 60µl of both Firefly and Gaussia were added to their respective triplicates and then measured using the Victor™ light luminescence counter. The relative luciferase activity was then determined by calculating the ratio of Gaussia and Firefly counts. The miR-494 effects with and without oligonucleotide blockers were determined by normalising each relative luciferase activity to their respective negative control samples (miR-NC).

3.4. RNA Extraction

3.4.1 Transfection of HuH-7 cells with Oligonucleotides for RNA extraction

HuH-7 cells were trypsinised (section 3.1.1.) and the concentration and cell viability was measured using the Countess Automated Cell Counted. Cells were then seeded into 24 well plates at a density of $1.5x10^5$ cells/well. The seeded plate was then incubated at 37˚C/5% CO_2 for 12 hours to allow cells to adhere before transfection. The following day, cells were checked for optimum confluency, being ~70-80%.

Transfection complexes were formed by combining 1.5 µl of Lipofectamine™ 3000 and 4µl of 100µM 2'OMe into 48.5µl of Opti-MEM™. Complexes were

allowed to form through a 15 minute incubation period 20-25ºC. After incubation, the entire volume was transferred into a new tube containing 946µl of Opti-MEM™, resulting in an initial 2'OMe concentration of 400nM. Consecutive 1:2 dilutions were performed with 500µl of transfection complexes and 500µl of Opti-MEM™, resulting in 3 final concentrations of 400nM, 200nM and 100nM of 2'OMe complexes.

The media was removed from the seeded cells and then replaced with 500µl of each concentration of transfection complex. The plate was incubated for 12 hours at 37ºC/ 5% CO_2.

3.4.2. Total RNA Extraction

The complete growth media was removed from the transfected cells which were then rinsed with ~1mL of cold PBS. Cells were then lysed directly in their wells through the addition of 1mL of TRIzol™ reagent per 3.5cm diameter well and scraped off the surface of each well before being added into new 2mL tubes. 0.25mL of chloroform per 1mL of TRIzol™ reagent was then added and each tube was vortexed/ shaken vigorously for 15-30 seconds before being incubated at 20-25ºC for 3 minutes. Each sample was then centrifuged at 12000 x g and 2ºC for 15 minutes using the Beckman Coulter Allegra™ 25R centrifuge. Centrifuging of the samples resulted in the mixture separating into a lower red phenol:chloroform organic phase an interphase and an upper colourless aqueous phase. 600µl of the aqueous phase containing RNA was carefully removed and transferred into a new 2mL tube for RNA precipitation. 0.5mL of isopropyl alcohol per 1mL of TRIzol™ reagent was added to each tube. The samples were incubated for 10 minutes at 20-25ºC and then centrifuged at 12000 x g and 4ºC for 10

minutes. The centrifuge step was repeated if a pellet was not visible on the bottom of the tube. Once a pellet was visible, the supernatant was removed completely, and the pellet was washed with 1mL of 75% ethanol per 1mL TRIzol™ reagent that was previously used. The samples were then centrifuged at 7500 x g and 2ºC for 5 minutes and the ethanol removed. This washing step was repeated once more, and all ethanol was removed from the pellet. The pellet was left to air-dry for ~10 minutes, it was then dissolved in dH_2O and the concentration of RNA was determined using Nanodrop® 1000 spectrophotometer. Each sample was then diluted to 1000ng/µl using nuclease free water and stored at -80ºC until needed.

3.5. Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

3.5.1. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was converted into complementary DNA (cDNA) using the SuperScript® III Reverse Transcriptase (RT) Enzyme. For first strand synthesis, 1µl of 10mM dNTP, 1 µl of 50µm random hexamers, 1µl of nuclease free water and 10µl of 1000ng/µl total RNA were added to a nuclease free microcentrifuge tube. The samples were then placed in the Bio-Rad Real-Time PCR Detection System and incubated at 65ºC for 5 minutes. Following incubation, the samples were then placed on ice for a minimum of 1 minute. A master mix was prepared for cDNA conversion, this included 4µl of 5x first strand buffer, 1µl of 0.1M DTT, 1µl of RNaseOUT™ Recombinant and 1ul of 200U/µl Superscript III RT enzyme per sample. The 7µl mixture was added to the first strand synthesis product and then placed in the Bio-Rad Real-Time PCR Detection System and cycled at 50ºC for 60

minutes followed by 70ºC for 15 minutes then held at 4ºC. The cDNA was then used immediately for qPCR or stored at 4ºC.

3.5.2. Quantitative Polymerase Chain Reaction (qPCR)

The relative amount of *PROS1* expression was determined using the TaqMan® Gene Expression assay and normalised by using β-Actin (ACTB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous housekeeping genes. Each master mix was prepared by adding 0.5ul of 20x TaqMan® gene expression assay to 5ul of 2x universal master mix per required well. This master mix was vortexed thoroughly before 5.5ul was loaded into respective wells on a 96 or 384 well PCR reaction plate, depending on the number of samples. The 20ul cDNA was diluted 1:7 with RNase free water and 4.5ul of each sample was added to their respective wells in triplicates. The PCR plate was then placed into the Bio-Rad CFX 96 or CFX 384 Real Time PCR Detection System and cycled under the following conditions: 95C for 10 minutes, 40 cycles at 90C for 15 seconds, followed by 60C for 1 minute. The relative fold changes to the mRNA levels were calculated via the 2-ΔΔCT method.

3.6. Antisense Oligonucleotide Synthesis and Modifications

ASO sequences were designed to span the 3 PROS1 miR-494 binding sites, with additional flanking sequence. ASOs were synthesised and provided by Rakesh Veedu from CCG, Murdoch. All modified ASOs were prepared in-house on GE AKTA oligopilot 10 oligonucleotide synthesizer via standard phosphoramidite chemistry in 1 μmol scale. Synthesized oligonucleotides

were deprotected and cleaved from the solid support by treatment with NH_4OH at 55˚C for 12 hours. The crude oligonucleotides were then purified, desalted and verified by MALDI-ToF MS analysis.

Sequences for the three ASOs used are as follows:

Site 1 → 5’ ATTGAAACATAAG 3’

Site 2 → 5’ CAAAAACATAAAC 3’

Site 3 → 5’ GTGAAACATCTG 3’

All 3 ASOs were initially synthesised with 2’OMe modifications, however sites 2 and 3 had additional LNA modifications.

A reverse complement LNA ASO of site 2 was synthesised to be used as a loading control in the miR-494 positive control samples.

Reverse Complement → 5’ GTTTATGTTTTTG 3’

3.7. Statistical Analysis

To analyse the data found in the luciferase reporter assays, a two-tailed Student’s *t*-test with unequal variance was performed. A *p*-value of <0.05 was set to show statistical significance.

To analyse the data found in the qRT-PCR, the Bio-Rad CFX 96 or CFX 384 Real Time PCR Detection System used an internal 2-ΔΔCT method. A two-tailed Student’s *t*-test with unequal variance was then performed with a *p*-value of <0.05 set to show statistical significance.

Chapter 4: Results

4.1. Optimisation of Lipid Transfection

4.1.1. Metafectene® Pro vs Lipofectamine™ 3000

To ensure optimum conditions for transfection, Metafectene® Pro was tested against Lipofectamine™ 3000. This was done to determine which lipofection reagent provided the most efficient lipid uptake into the HuH-7 cells. This would consequently lead to the highest detectable signal of relative luciferase activity when cells were co-transfected with the pRR-5-duo luciferase REPORT vector and 50nM pre-miR-494. Respective protocols were carried out (Sections 3.3.3 and 3.3.4) and relative luciferase activity was measured 24 hours post transfection. Each independent pre-miR-494 sample had an identical negative control sample to normalise against. Treatment of cells with Metafectene® Pro resulted in a ~24% decrease in luciferase activity, however this was not statistically significant (p =0.7), while treatment of cells with Lipofectamine™ 3000 resulted in a ~30% decrease in luciferase activity (p <0.05) (Fig. 4.1)

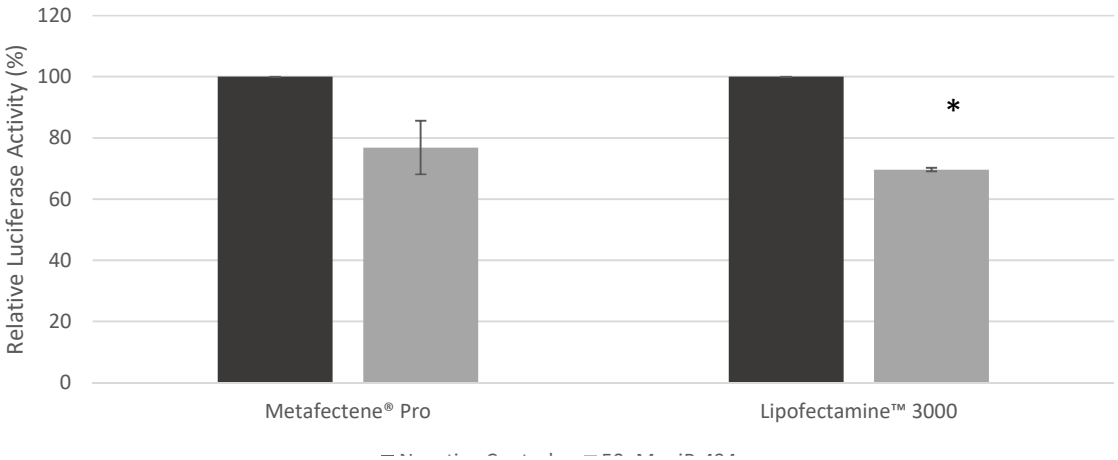


Figure 4.1. HuH-7 cells were co-transfected with pRR-5-Duo-REPORT luciferase construct and 50nM of miR-NC or miR-494 precursors. Protocols corresponding to each reagent were carried out and luciferase activity was measured 24 hours post transfection (* p <0.05) Results are from 3 independent transfections.

4.1.2. Lipofectamine™ 3000 – P3000™ reagent vs no P3000™ reagent

Optimisation of the Lipofectamine protocol meant determining whether using the P3000™ reagent that came with the kit would allow greater lipid uptake into the cells or interfere with the miRNA. The Lipofectamine™ 3000 protocol (Section 3.3.4) was carried out with half of the samples altered to not include the P3000™ reagent (Fig. 4.2). Each independent pre-miR-494 sample had an identical negative control sample to normalise against. Treatment of cells with Lipofectamine™ 3000 and P3000™ reagent resulted in a ~30% decrease in relative luciferase activity, however this is not statistically significant (p = 0.3). Treatment of cells with Lipofectamine™ 3000 only resulted in ~30% increase in relative luciferase activity, this was however not statistically significant (p = 0.7).

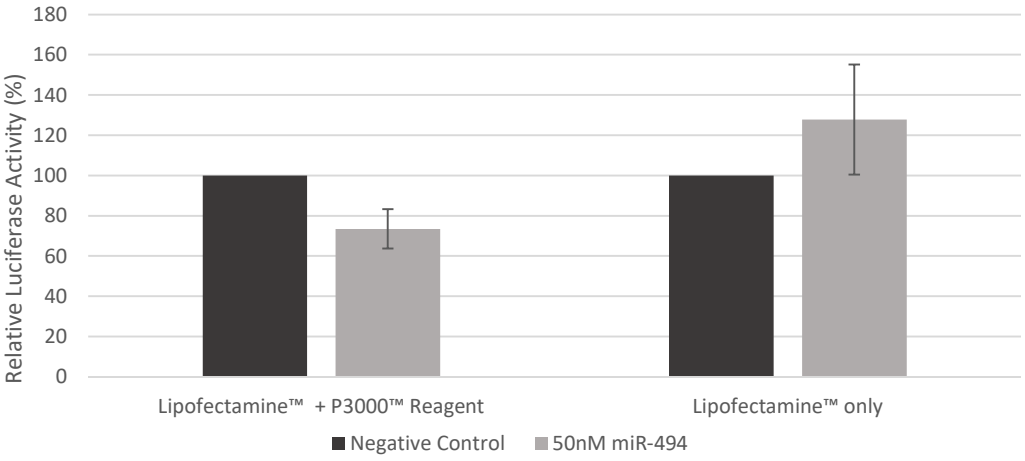


Figure 4.2. HuH-7 cells were co-transfected with pRR-5-Duo-REPORT luciferase construct and 50nM of miR-NC or miR-494 precursors. Lipofectamine™ 3000 was tested with and without the P3000 reagent and luciferase activity was measured 24 hours post transfection (* p <0.05). Results are from 3 independent transfections.

4.1.3. miR-494 Dose Response

A dose response transfection was done to determine the optimum concentration of miR-494 to be used in further experiments (Fig 4.3.). HuH-7 cells were co-transfected with the pRR-5-Duo luciferase REPORT vector and concentrations of 5nM, 10nM, 50nM and 100nM pre-miR-494. The Lipofectamine™ 3000 protocol was carried out and relative luciferase activity was measured 24 hours post transfection. Each independent pre-miR-494 sample had an identical negative control sample to normalise against. The relative luciferase activity for each concentration was 48%, 51%, 50.5% and 48.5% respectively. 50nM was consequently chosen for further experiments.

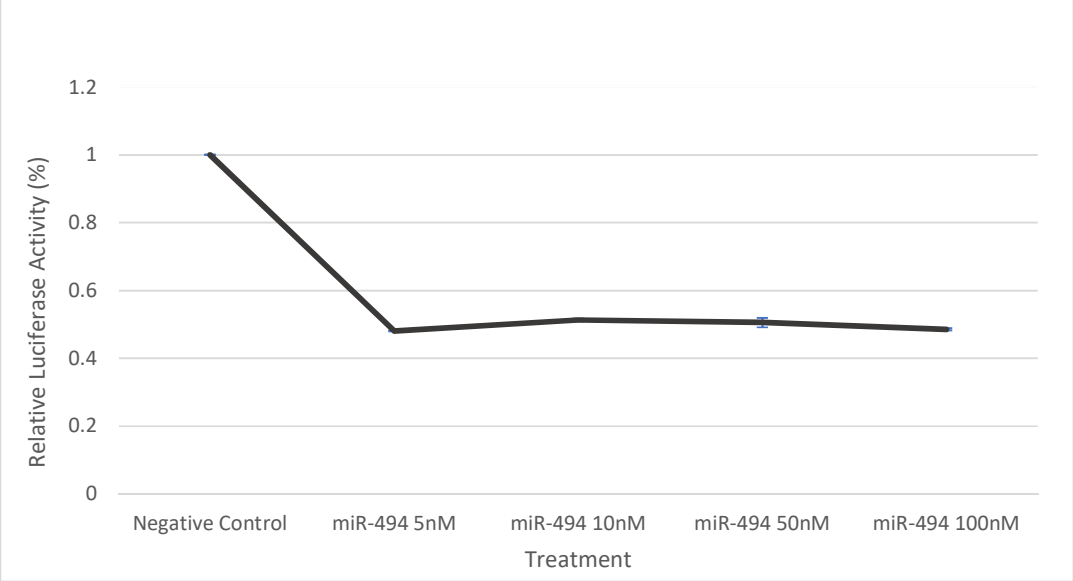


Figure 4.3. HuH-7 cells were co-transfected with pRR-5-Duo-REPORT luciferase construct and 5nM, 10nM, 50nM and 100nM of miR-494. Relative luciferase activity was measured and independent miR-494 samples were normalised against a negative control. Results are from 3 independent transfections.

4.2. Effects of miR-494 with and without oligonucleotides

4.2.1. Relative luciferase activity after lipid transfection

To determine the effects of miR-494 with and without 2'OMe or LNA oligonucleotides, HuH-7 cells were transfected as per the previously established Lipofectamine™ 3000 protocol. Each independent pre-miR-494 sample had an identical negative control sample to normalise against. Cells transfected with pre-miR-494 had a ~30% decrease in luciferase activity (p <0.05). Additionally, cells were transfected with 50nM oligonucleotides as follows: Site 2 - LNA, Site 1 - 2'OMe, Site 2 - 2'OMe, Site 3 - 2'OMe and Site 3 - LNA. When either 2'OMe or LNA oligonucleotides were added, luciferase activity increased. This was however not statistically significant when analysed with a Student's t-test (p >0.05) (Fig. 4.4.).

The two modified ASOs were compared against each other to determine which provided the greatest increase in luciferase activity. It was seen that when transfected with the 2'OMe modified ASO at site 3 the relative luciferase activity was calculated to be ~78%, showing an increase of ~10% in comparison to the miR-494 only sample. This was compared to the LNA modified site 3, which showed a calculated relative luciferase activity of ~94%, showing an increase of ~25% when compared to the miR-494 only sample (Fig. 4.4. B.).

The effects of pre-miR-494 with and without the various combinations of 2'OMe or LNA oligonucleotides targeting the 3 sites found on the *PROS1* 3'UTR was determined through the transfection of HuH-7. Each independent pre-miR-494 sample had an identical negative control sample to normalise

against. Cells transfected with pre-miR-494 only had a ~30% decrease in luciferase activity, however not statistically significant. Additionally, cells were transfected with 50nM oligonucleotides in combinations as follows: Site 1 - 2'OMe and Site 2 - LNA, Site 1 - 2'OMe and Site 3 - LNA, Site 2 - LNA and Site 3 - LNA and Site 1 - 2'OMe, Site 2 - LNA and Site 3 - LNA. When combinations of 50nM oligonucleotides were added, luciferase activity increased. This was however not statistically significant when analysed with a Student's *t*-test ($p > 0.05$) (Fig. 4.5.).

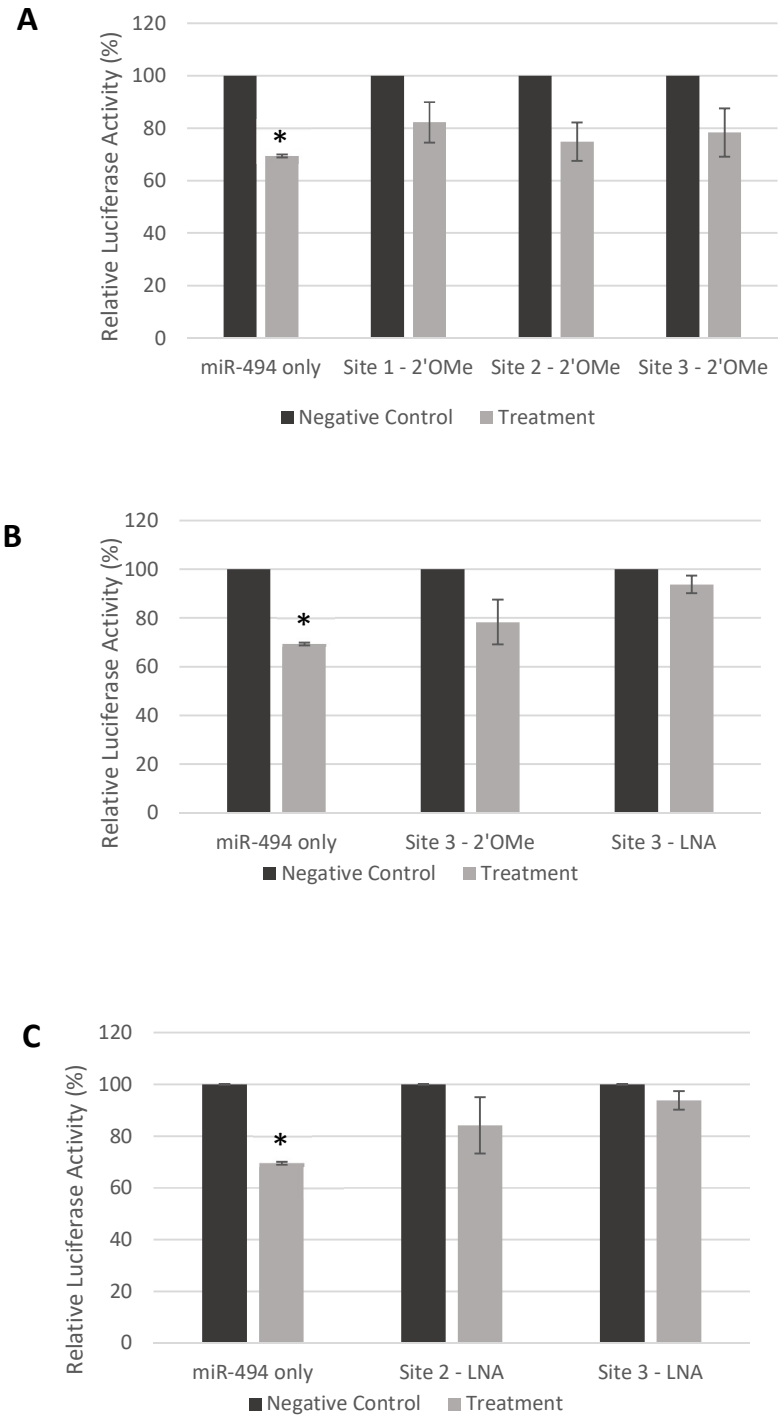


Figure 4.4. HuH-7 cells were co-transfected with pRR-5-Duo-REPORT luciferase construct and 50nM of miR-NC or miR-494 precursors. (A) The cells were then treated with 50nM 2'OMe oligonucleotides, (B) Comparing 50nM 2'OMe oligonucleotide and 50nM LNA modified oligonucleotides at site 3 and (C) The cells were treated with 50nM LNA modified oligonucleotides. Luciferase activity was measured 24 hours post-transfection. (* p <0.05). Results are from 3 independent transfections.

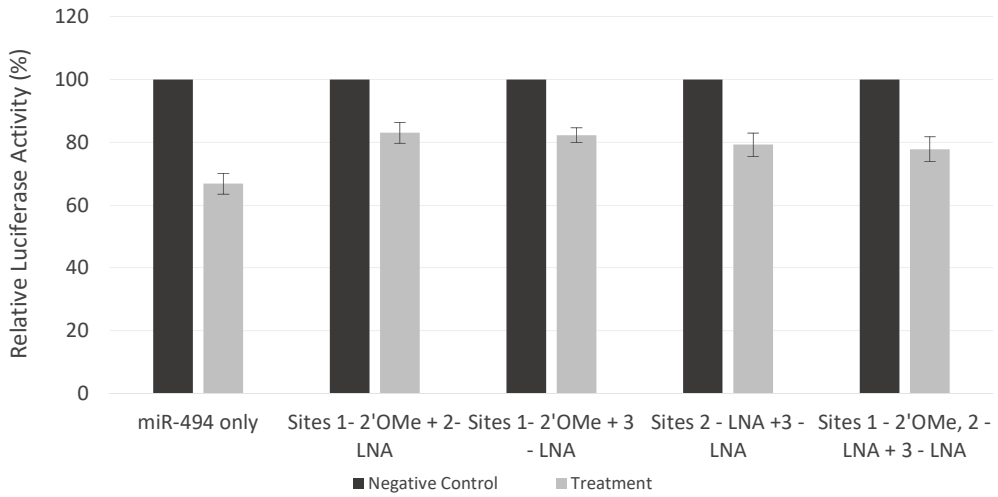


Figure 4.5. HuH-7 cells were co-transfected with pRR-5-Duo-REPORT luciferase construct and 50nM of miR-NC or miR-494 precursors. The cells were then treated with combinations of 50nM 2'OMe oligonucleotides or LNA modified oligonucleotides and luciferase activity was measured 24 hours post-transfection. (* p <0.05). Results are from 3 independent transfections.

4.2.2. mRNA levels of *PROS1* in 2'OMe or LNA transfected HuH-7 cells with and without miR-494

To determine mRNA levels of *PROS1*, a TaqMan® Gene Expression Assay (Section 3.5.2) was used in three independent transfections RNA was extracted from HuH-7 cells transfected with 50nM pre-miR-494 and 50nM of Site 1 – 2'OMe, Site 2 – LNA, Site 3 – LNA or Site 2 + 3 – LNA. After conversion to cDNA, Gene Expression analysis was done. After normalising to housekeeping genes, β-Actin (ACTB) and glyceraldehyde 3-phosphate (GAPDH) using the 2-ΔΔCT method, mRNA levels of samples with oligonucleotide blockers were compared to a miR-494 only control sample (Fig. 4.6, A). The mRNA levels of *PROS1* with site 2, site 3 and site 2 + 3 in combination (all LNA oligonucleotides) show a significant increase when

compared with the control (p<0.01). Site 1 2'OMe does not show a significant change in *PROS1* mRNA (p = 0.2).

PROS1 mRNA levels were also determined without the addition of exogenous miR-494. TaqMan® Gene Expression Assay was used in three independent transfections. RNA was extracted from HuH-7 cells transfected with either 50nM or 100nM of site 2 LNA or 2'OMe oligonucleotides. After conversion to cDNA, gene expression analysis was done. After normalising to housekeeping genes, β-Actin (ACTB) and glyceraldehyde 3-phosphate (GAPDH) using the 2-ΔΔCT method, mRNA levels of samples with oligonucleotide blockers were compared to the control sample which contained the scrambled ASO control (Fig. 4.6, B). The site 2 – 2'OMe transfected samples at both concentrations result in a decrease of *PROS1* mRNA levels when compared with the control, however there is no statistical significance (p = 0.1 (100nM), p= 0.3 (50nM)). The site 2 – LNA transfected samples at both concentrations result in a slight increase of *PROS1* mRNA levels when compared with the control, however there was no statistical significance (p = 0.8 (100nM), p= 0.7 (50nM)).

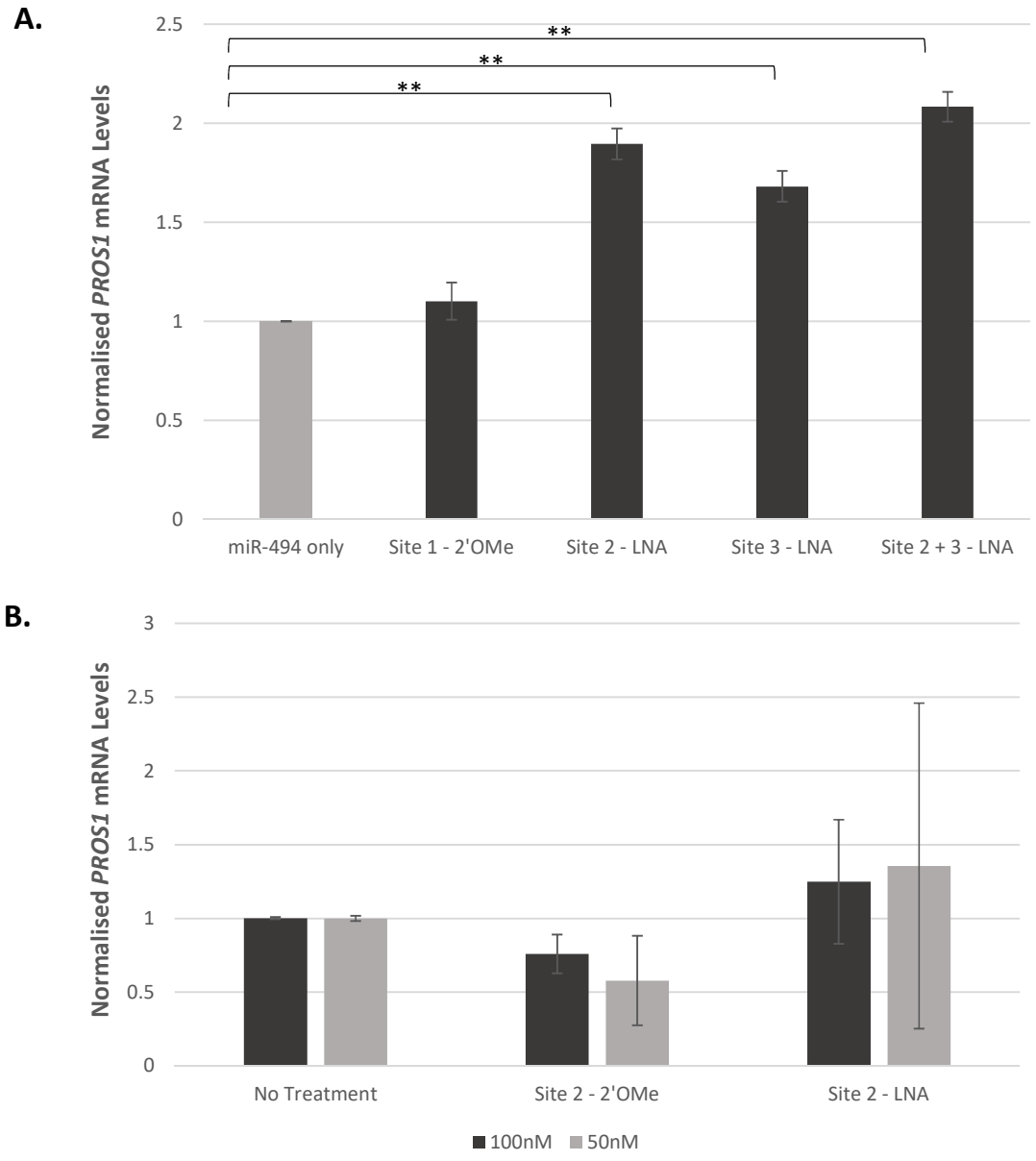


Figure 4.6. HuH-7 cells were transfected with 50nM oligonucleotides specific to the 3 sites found within the *PROS1* 3'UTR. *PROS1* levels were quantified by quantitative real-time PCR (qRT-PCR). Analysis of *PROS1* expression was normalised with β-Actin (ACTB) and glyceraldehyde 3-phosphate (GAPDH) using the 2-ΔΔCT method. A. with miR-494, B. without miR-494. (* p <0.05, ** p<0.01)

Chapter 5: Discussion

The miRNA of interest in this project, miR-494, has various roles through the targeting of numerous genes which include cell cycle regulators, regulators of DNA replication and transcription factors (Tay et al. 2016). This project focussed on the role miR-494 has in the regulation of PS when binding to three functional sites found within the *PROS1* mRNA 3'UTR. The role of miR-494 in PS regulation has been poorly characterised with only one study by Tay et al. (2013) examining this regulation in patients with an acquired PS deficiency. The study investigated PS deficiency in association with elevated circulating oestrogen levels that came about as a result of pregnancy, oral contraceptives and oestrogen replacement therapy. In comparison, this project looked at hereditary PS deficiency associated with autosomal dominant mutations found within the *PROS1* gene. These mutations are mostly heterozygous, or in rare cases, double heterozygous. This is because the homozygous form usually presents in neonates with purpura fulimans (Khan and Dickerman 2006). The heterozygous mutation allows the potential for therapy to be directed at the intact *PROS1* wild type allele.

The results found in this project show that miR-494 downregulates the expression of *PROS1* and the addition of specifically designed modified oligonucleotides can negate the effect miR-494 has on *PROS1* mRNA 3'UTR regulation. This discussion will offer possible reasons behind results obtained in this project as well as provide improvements and additional direction for further studies.

5.1. Optimisation of Lipid Transfection

Two lipid based transfection reagents were tested to determine which had the greatest nucleic acid uptake into the HuH-7 cells, translating to the most consistent luciferase activity that results in the greater decrease when miR-494 was transfected. Lipofectamine™ 3000 consistently resulted in high luciferase activity when compared to Metafectene® Pro and was therefore chosen for subsequent experiments.

Additionally, Lipofectamine™ 3000 contained a P3000™ reagent that was not recommended for use with small interfering RNAs (siRNA). As miRNAs and siRNAs share many similarities, both being short duplex RNA molecules that target mRNA (Lam et al. 2015) the transfection was tested with and without the addition of this reagent. The results showed that the addition of this reagent increased the transfection performance compared to no reagent and was therefore used in all further transfections.

Finally, a dose response using increasing concentrations of miR-494 was done to ensure the optimum concentration was chosen. It was seen that all concentrations could result in a ~50% decrease in luciferase activity, which correlates to *PROS1* 3'UTR expression being downregulated. However, 50nM was chosen to be the optimum concentration, this was to ensure all miR-494 binding sites were completely saturated. The 100nM concentration was not chosen as the pRR-5-Duo plasmid and ASOs were to be co-transfected with miR-494 and high concentrations of DNA and RNA being transfected into cells can result in cytotoxicity (Romøren et al. 2004).

5.2. Principle Findings

Overall, the luciferase assay results suggest that the use of ASOs targeted to the 3'UTR of *PROS1* mRNA can block the miR-494 binding interaction leading to increased expression. Initially 2'OMe phosphorothioate ASOs were tested, as this modification provides protection from exonuclease attack, allowing the ASOs to bind to their corresponding target within the 3'UTR of *PROS1* without rapid degradation. It was seen that the addition of 2'OMe ASOs at all three sites mildly negated the effect miR-494 has on the regulation of the *PROS1* 3'UTR.

Following initial independent transfections of the three 2'OMe ASOs (Appendix - Fig. Appx 1.), it was seen that sites 2 and 3 resulted in the greatest increase of relative luciferase activity when compared to site 1. Because of this, LNA modified versions of these ASOs were made. This modification contains a methylene bridge that connects the 2'-oxygen of the ribose with the 4'-carbon, increasing the affinity these ASOs have for complementary sequences (Fig. 5.1.) (Braasch and Corey 2001). A comparison was done between site 3 -2'OMe and site 3 – LNA to show that the LNA modification results in a more pronounced increase in relative luciferase activity when compared to the miR-494 sample alone and when compared to the 2'OMe modification. This increase in luciferase activity correlates to a negating effect on miR-494 regulation of the *PROS1* 3'UTR. Furthermore, the site 2 – LNA also showed a pronounced increase in relative luciferase activity when compared to the miR-494 sample alone.

As mentioned, these results show a considerable trend toward *PROS1* expression being increased with the addition of these modified ASOs,

particularly LNA modified. The results however did not show any significance. There were previous inconsistencies with luciferase assay results, with independent transfections varying in relative luciferase activity for the sample with miR-494 only (Appendix – Fig. Appx 1). Luciferase activity would vary from a ~10% decrease to ~50%, with the 10% decrease not being enough to compare to the addition of ASOs. This very low effect of miR-494 could correlate to the HuH-7 cells that were transfected. If cells were at a high passage number, they began to exhibit cytoplasmic extensions and resulted in less efficiency of transfection. It was determined that a passage number of 20 should be the maximum before cells began to become less efficient for transfection.

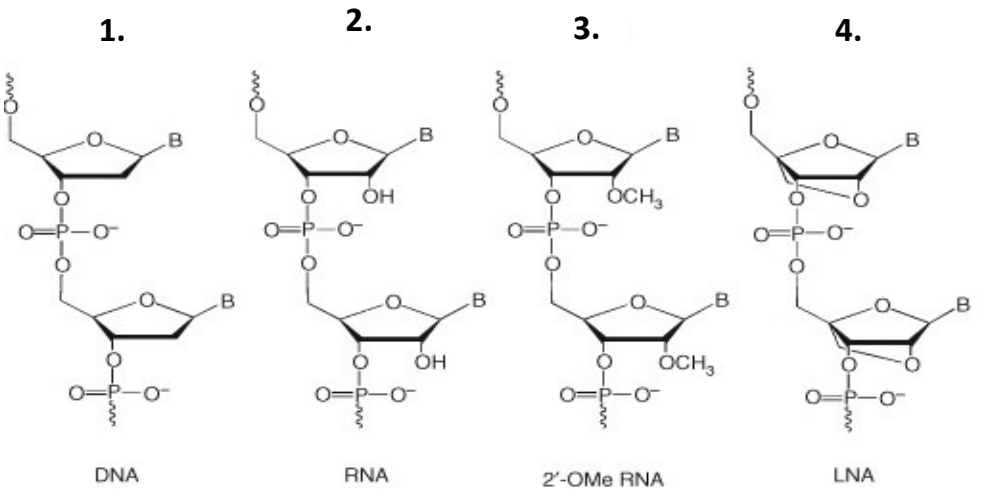


Figure 5.1. Chemical structure of (1) DNA, (2) RNA, (3) 2'OMe and (4) LNA . (Devi et al. 2015)

To validate the trend of increased expression with the addition of modified ASOs, *PROS1* mRNA levels were determined through qPCR. The initial

results showed that *PROS1* mRNA was significantly increased when LNA modified ASOs were introduced to either site 2 or site 3. This significance also occurred when a combination of both site 2 and 3 – LNA was introduced, resulting in a 2-fold increase in *PROS1* mRNA when compared to miR-494 alone. There was however, no significance in the mRNA level when transfected with the site 1 – 2'OMe. The level of mRNA is considerably lower in comparison to the LNA ASOs, this is due to the greater nuclease resistance seen with the LNA modification, allowing greater protection of the *PROS1* mRNA transcript. 2'OMe ASOs also exhibit a lessened binding affinity and a lower T_m compared to the LNA modifications. It has been seen that 2'OMe modifications only result in a slight increase in T_m, by less than 1°C per modified oligonucleotide, compared to 1.5-4°C per LNA monomer introduced into an 18mer oligonucleotide (Kurreck et al. 2002).

PROS1 mRNA levels after transfection with modified ASOs in the presence of endogenous miR-494 was also determined. This compared 100nM and 50nM of 2'OMe and LNA ASOs targeted to site 2. The concentration of 100nM was tested in addition to 50nM as the 2'OMe ASOs have a lowered binding affinity, therefore an increase in concentration would provide complete saturation of the target site. It was seen that at both concentrations, the site 2 – 2'OMe resulted in a lowered *PROS1* mRNA. This lowered level of mRNA could be a result of nuclease degradation of the ASOs before binding, preventing the regulatory action of endogenous miR-494. Additionally, it was previously reported that the charged phosphorothioate backbone results in an increase of solubility (Braasch and Corey 2001), this could also account for the lowered mRNA levels. Both concentrations of site

– 2 LNA exhibited increased *PROS1* mRNA levels, however there was no statistical significance as well as a large error bar present when looking at 50nM. The lack of significance and large error bars could be due to possible degradation once again as well as a degree of resistance. However, it is evident that the LNA modification shows the desired effect compared to 2'OMe.

5.3. Future Direction

Data from this preliminary study has provided evidence that the use of modified ASOs specific to the three sites found within the *PROS1* 3'UTR can negate the downregulatory effect miR-494 has on PS expression. This data will provide the basis for a more in-depth analysis of the use of these ASOs that could result in the novel therapeutic application for hereditary PS deficiency and ultimately for use *in vivo*.

5.3.1. ASO modifications for *in vitro* analysis

As mentioned, the LNA modified ASO was only produced for sites 2 and 3. Due to time constraints, a site 1 – LNA was not able to be synthesised and tested. An LNA modified ASO for site 1 would be important for further luciferase assay and qPCR results for the singular site. In addition, this would provide more reliable combination results as all ASOs would have equal modifications.

5.3.2. ASO combination analysis

The analysis of ASOs in combination was conducted using a luciferase assay, however this will need to be repeated once a site 1 – LNA is synthesised, to provide more reliable results. qPCR analysis of these combinations will also need to be completed to determine the *PROS1* mRNA levels, followed by Western Blot analysis. This is an important next step as it was seen that the combination of site 2 and 3 – LNA resulted in a significant increase in *PROS1* mRNA compared to the singular ASOs. Therefore, additional combinations require the same investigation.

5.3.3. Western Blot Analysis

Protein analysis by western blot was not conducted in this preliminary project due to time constraints. An important next step would be the analysis of PS secreted into culture medium after HuH-7 cells are treated with miR-494 and both singular and combinations of ASOs. This would provide further confirmation to the qPCR results, particularly if a significant increase in PS levels are seen. This analysis is essential to provide support for the results obtained through qPCR analysis.

5.3.4. Experimental changes

Luciferase assay results did not provide any significance regardless of distinct differences seen in relative luciferase activity. Additionally, the replicates were not always consistent, resulting in no statistical significance when analysed with a Student's *t*-test. To provide more evidence toward the use of ASOs as miR-494 binding inhibitors, more replicates will be needed. To ensure replicates remain consistent, HuH-7 cells should be maintained at low passage numbers, with a maximum of 20.

PROS1 mRNA analysis in the presence of endogenous miR-494 also did not provide any significance. Future endogenous miR-494 analysis should be conducted with oestrogen treated HuH-7 cells. It has been previously discovered that oestrogen upregulates miR-494 expression (Tay et al. 2013). This treatment will provide a more reliable result compared to non-treated cells.

5.3.5. ASO modifications for *in vivo* analysis

Currently, the ASOs that have been used in this project have a phosphorothioate backbone. These linkages have been shown to have high nuclease stability, however *in vivo* evaluation has demonstrated that this modified backbone exhibits several toxicities (Wada et al. 2016), with the most evident being transient activation of the complement cascade (Iannitti, Morales-Medina, and Palmieri 2014). The addition of the 2'OMe modification to this backbone provides a slight protection against non-specific binding and increases the stability of binding to a target, however in long-term experiments and *in vivo* applications, enzymatic degradation still remains a significant problem due to the negatively charged backbone. Additionally, the sulphur atom that replaces a non-bridging oxygen in the phosphorothioate backbone provides this ASO with the potential to produce non-specific effects with proteins within a cell (Summerton 2003; Kurreck 2003). The addition of the LNA modification provides significantly more protection against nuclease degradation and increased affinity for complementary sequences (Kauppinen, Vester, and Wengel 2005), however the negatively charged backbone lessens this nuclease protection as well as producing toxic effects *in vivo*. The LNA ASOs used in this project have provided more

pronounced results compared to the 2'OMe ASOs, and should therefore continue to be tested *in vitro*.

For future *in vivo* tests, the LNA modification on a phosphorothioate backbone isn't adequate due to toxicity and long-term degradation (Summerton 2003). An alternative for this is a morpholino. The morpholino backbone is non-ionic, therefore does not result in non-specific binding, reducing the toxicity of this ASO (Kurreck 2003). Additionally, morpholinos have high stability and specificity in biological systems (Summerton 2003). Further modifications suggested for *in vivo* use of morpholinos include conjugation with lipophilic compounds such as cholesterol, or cell penetrating peptides such as lysine. This conjugation is recommended as morpholinos have been seen to have poor cellular uptake alone (Winkler 2013).

5.3.6. Implications

Successful inhibition of the miR-494/ *PROS1* mRNA 3'UTR interaction, as shown through preliminary tests in this project, may ultimately be developed as a novel therapeutic strategy for patients with an endogenous protein deficiency. This project focussed on PS deficiency, but it will be possible to use this strategy for additional diseases resulting from a protein deficiency. The ASOs used in this project will be able to be tested in other protein deficiency diseases that result from a heterozygous mutation. The results seen in this project have shown that ASOs are successful in the prevention of miRNA regulation, allowing the functional allele to increase expression. Therefore, the main implication of this project is that specifically designed ASOs will be able to be used to bypass a mutation that results in protein

deficiency, allowing an individual's functional allele to have its expression boosted.

5.4. Conclusion

To conclude, the role of miR-494 as a regulator of *PROS1* expression has been poorly characterised, with only one study suggesting direct regulation of *PROS1* mRNA is through the binding of miR-494 to three functional sites found within the 3'UTR. The results in this project have further characterised miR-494 as a down regulator of *PROS1* mRNA expression. Ultimately, this project aimed to inhibit this downregulation through the use of specifically designed ASOs, this principle was validated through luciferase assay and qPCR analysis. These findings provide the basis for a novel therapeutic approach to treating endogenous protein deficiencies. Through further testing and additional modifications of ASOs, this treatment can potentially bypass an individual's mutation that is causative for a disease by increasing the expression of their functional allele.

References

Alberts, Bruce. 2002. *Molecular biology of the cell* (Garland Science: New York).

Anderson, F. A., Jr., and F. A. Spencer. 2003. 'Risk factors for venous thromboembolism', *Circulation*, 107: I9-16.

Andersson, Helena M., Márcia J. Arantes, James T. B. Crawley, Brenda M. Luken, Sinh Tran, Björn Dahlbäck, David A. Lane, Suely M. Rezende, Malmö Clinical Chemistry, fakulteten Medicinska, Medicine Faculty of, Malmö Klinisk kemi, universitet Lunds, Medicine Department of Translational, University Lund, and medicin Institutionen för translationell. 2010. 'Activated protein C cofactor function of protein S: A critical role for Asp95 in the EGF1-like domain', *Blood*, 115: 4878-85.

Austin, Steven K. 2017. 'Haemostasis', *Medicine (United Kingdom)*, 45: 204-08.

Bandiera, Simonetta, Sébastien Pfeffer, Thomas F. Baumert, and Mirjam B. Zeisel. 2015. 'miR-122 – A key factor and therapeutic target in liver disease', *Journal of Hepatology*, 62: 448-57.

Barta, T., L. Peskova, and A. Hampl. 2016. 'miRNAsong: a web-based tool for generation and testing of miRNA sponge constructs in silico', *SCIENTIFIC REPORTS*, 6: 36625.

Baumann, V., and J. Winkler. 2014. 'miRNA-based therapies: strategies and delivery platforms for oligonucleotide and non-oligonucleotide agents', *FUTURE MEDICINAL CHEMISTRY*, 6: 1967-84.

Black, Douglas L. 2003. 'MECHANISMS OF ALTERNATIVE PRE-MESSENGER RNA SPLICING', *Annual Review of Biochemistry*, 72: 291-336.

Bose, Debojit, Gopal Jayaraj, Hemant Suryawanshi, Prachi Agarwala, Subrata Kumar Pore, Rajkumar Banerjee, and Souvik Maiti. 2012. 'The tuberculosis drug streptomycin as a potential cancer therapeutic: Inhibition of mir-21 function by directly targeting its precursor', *Angewandte Chemie - International Edition*, 51: 1019-23.

Bouchie, A. 2013. 'First microRNA mimic enters clinic', *NATURE BIOTECHNOLOGY*, 31: 577-77.

Braasch, Dwaine A., and David R. Corey. 2001. "Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA." In, 1-7. England: Elsevier Ltd.

Brennecke, Julius, Alexander Stark, Robert B. Russell, and Stephen M. Cohen. 2005. 'Principles of microRNA-target recognition', *PLoS biology*, 3: e85.

Brouwer, Jan-Leendert P., Willem M. Lijfering, Min Ki Ten Kate, Hanneke C. Kluin-Nelemans, Nic J. G. M. Veeger, and Jan Van Der Meer. 2009. 'High long-term absolute risk of recurrent venous thromboembolism in patients with hereditary deficiencies of protein S, protein C or antithrombin', *THrombosis and Haemostasis*, 101: 93-99.

Brouwer, Jan-Leendert P., J. G. M. Veeger Nic, Schaaf Wim van der, Hanneke C. Kluin-Nelemans, and Meer Jan van der. 2005. 'Difference in absolute risk of venous and arterial thrombosis between familial protein S deficiency type I and type III. Results from a family cohort study to assess the clinical impact of a laboratory test-based classification', *British Journal of Haematology*, 128: 703-10.

Brown, Brian D., Alessio Cantore, Andrea Annoni, Lucia Sergi Sergi, Angelo Lombardo, Patrizia Della Valle, Armando D'Angelo, and Luigi Naldini. 2007. 'A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice', *Blood*, 110: 4144-52.

Carrington, James C., and Victor Ambros. 2003. 'Role of MicroRNAs in Plant and Animal Development', *Science*, 301: 336-38.

Caspers, Michael, Anna Pavlova, Julia Driesen, Ursula Harbrecht, Robert Klamroth, Janos Kadar, Ronald Fischer, Bettina Kemkes-Matthes, and Johannes Oldenburg. 2012. 'Deficiencies of antithrombin, protein C and protein S - practical experience in

genetic analysis of a large patient cohort', *THrombosis and Haemostasis*, 108: 247-57.
Chesnoy, S., and L. Huang. 2000. 'STRUCTURE AND FUNCTION OF LIPID-DNA COMPLEXES FOR GENE DELIVERY', *Annual Review of Biophysics and Biomolecular Structure*, 29: 27-47.
Christopher, Ajay Francis, Raman Preet Kaur, Gunpreet Kaur, Amandeep Kaur, Vikas Gupta, and Parveen Bansal. 2016. 'MicroRNA therapeutics: Discovering novel targets and developing specific therapy', *Perspectives in clinical research*, 7: 68-74.
Corey, D. R., and J. M. Abrams. 2001. 'Morpholino antisense oligonucleotides: tools for investigating vertebrate development', *GENOME BIOLOGY*, 2: REVIEWS1015-reviews15.3.
D'Angelo, A., S. Vigano-D'Angelo, C. T. Esmon, and P. C. Comp. 1988. 'Acquired deficiencies of protein S. Protein S activity during oral anticoagulation, in liver disease, and in disseminated intravascular coagulation', *Journal of Clinical Investigation*, 81: 1445-54.
D'Angelo, Armando, and Silvana Viganò D'Angelo. 2008. 'Protein S deficiency', *Haematologica*, 93: 498-501.
D'Angelo, Armando, Silvana Vigano D'Angelo, Patrizia Della Valle, Luciano Crippa, Elisabetta Pattarini, and Luigi Grimaldi. 1993. 'Autoimmune Protein S Deficiency in a Boy with Severe Thromboembolic Disease', *The New England Journal of Medicine*, 328: 1753-57.
Dahlbäck, B., B. Hildebrand, and J. Malm. 1990. 'Characterization of functionally important domains in human vitamin K-dependent protein S using monoclonal antibodies', *Journal of Biological Chemistry*, 265: 8127-35.
Day, D. A., and M. F. Tuite. 1998. 'Post-transcriptional gene regulatory mechanisms in eukaryotes: an overview', *Journal of Endocrinology*, 157: 361-71.
De Oliveira, Monica Cristina, Valérie Boutet, Elias Fattal, Didier Boquet, Jean-Marc Grognet, Patrick Couvreur, and Jean-Robert Deverre. 2000. 'Improvement of in vivo stability of phosphodiester oligonucleotide using anionic liposomes in mice', *Life Sciences*, 67: 1625-37.
De Stefano, V., P. Simioni, E. Rossi, D. Tormene, T. Za, A. Pagnan, and G. Leone. 2006. 'The risk of recurrent venous thromboembolism in patients with inherited deficiency of natural anticoagulants antithrombin, protein C and protein S', *Haematologica*, 91: 695.
Deutschman, Clifford S. 2005. 'Transcription', *Critical Care Medicine*, 33: S400-S03.
Devi, Gitali, Yuan Zhou, Zhensheng Zhong, Desiree-Faye Kaixin Toh, and Gang Chen. 2015. 'RNA triplexes: from structural principles to biological and biotech applications: RNA triplexes', *Wiley Interdisciplinary Reviews: RNA*, 6: 111-28.
Di Leva, Gianpiero, and Carlo Maria Croce. 2013. 'MicroRNAs', *Clinical biochemistry*, 46: 840-41.
Di Nisio, Marcello, Nick van Es, and Harry R. Büller. 2016. 'Deep vein thrombosis and pulmonary embolism', *The Lancet*, 388: 3060-73.
Diakos, Christofer, Sheng Zhong, Yuanyuan Xiao, Mi Zhou, Gisele M. Vasconcelos, Gerd Krapf, Ru-Fang Yeh, Shichun Zheng, Michelle Kang, John K. Wiencke, Maria S. Pombo-de-Oliveira, Renate Panzer-Grümayer, and Joseph L. Wiemels. 2010. 'TEL-AML1 regulation of survivin and apoptosis via miRNA-494 and miRNA-320a', *Blood*, 116: 4885-93.
Dias, Nathalie, and C. A. Stein. 2002. 'Antisense oligonucleotides: Basic concepts and mechanisms', *Molecular Cancer Therapeutics*, 1: 347-55.

Engesser, L., A. W. Broekmans, E. Briët, E. J. Brommer, and R. M. Bertina. 1987. 'Hereditary protein S deficiency: Clinical manifestations', *Annals of Internal Medicine*, 106: 677-82.

Esmon, C. 2003. 'The protein C pathway', *Critical Care Medicine*, 28: S44-S48.

Esmon, Charles T. 2000. 'The Anticoagulant and Anti-inflammatory Roles of the Protein C Anticoagulant Pathway', *Journal of Autoimmunity*, 15: 113-16.

Felekkis, K., E. Touvana, C. Stefanou, and C. Deltas. 2010. 'MicroRNAs: A newly described class of encoded molecules that play a role in health and disease', *Hippokratia*, 14: 236-40.

Felgner, Philip L., Thomas R. Gadek, Marilyn Holm, Richard Roman, Hardy W. Chan, Michael Wenz, Jeffrey P. Northrop, Gordon M. Ringold, and Mark Danielsen. 1987. 'Lipofection: A Highly Efficient, Lipid-Mediated DNA-Transfection Procedure', *Proceedings of the National Academy of Sciences of the United States of America*, 84: 7413-17.

Friedman, Robin C., Kyle Kai-How Farh, Christopher B. Burge, and David P. Bartel. 2009. 'Most mammalian mRNAs are conserved targets of microRNAs', *Genome Research*, 19: 92-105.

Gale, A. J. 2011. 'Continuing education course #2: current understanding of hemostasis', *Toxicol Pathol*, 39: 273-80.

Gandrille, S., D. Borgel, N. Sala, Y. Espinosa-Parrilla, R. Simmonds, S. Rezende, B. Lind, C. Mannhalter, I. Pabinger, P. H. Reitsma, C. Formstone, D. N. Cooper, H. Saito, K. Suzuki, F. Bernardi, M. Aiach, Subc Plasma Coagulation Inhibitors, Scientific Plasma Coagulation Inhibitors Subcommittee of the, Thrombosis Standardization Committee of the International Society on, and Haemostasis. 2000. 'Protein S deficiency: A database of mutations - Summary of the first update', *THrombosis and Haemostasis*, 84: 918-18.

García de Frutos, P., R. I. Alim, Y. Härdig, B. Zöller, and B. Dahlbäck. 1994. 'Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S', *Blood*, 84: 815.

García de Frutos, Pablo, Pablo Fuentes-Prior, Begoña Hurtado, and Núria Sala. 2007. 'Molecular basis of protein S deficiency', *THrombosis and Haemostasis*.

Garzon, Ramiro, Flavia Pichiorri, Tiziana Palumbo, Rodolfo Iuliano, Amelia Cimmino, Rami Aqeilan, Stefano Volinia, Darshna Bhatt, Hansjuerg Alder, Guido Marcucci, George A. Calin, Chang-Gong Liu, Clara D. Bloomfield, Michael Andreeff, and Carlo M. Croce. 2006. 'MicroRNA Fingerprints during Human Megakaryocytopoiesis', *Proceedings of the National Academy of Sciences of the United States of America*, 103: 5078-83.

Gill, Grace. 2001. 'Regulation of the initiation of eukaryotic transcription', *Essays in Biochemistry*, 37: 33-43.

Giri, T. K., B. O. Villoutreix, A. Wallqvist, B. Dahlbäck, and P. García De Frutos. 1998. 'Topological studies of the amino terminal modules of vitamin K-dependent protein S using monoclonal antibody epitope mapping and molecular modeling', *THrombosis and Haemostasis*, 80: 798-804.

Glass, Christopher K., and Michael G. Rosenfeld. 2000. 'The coregulator exchange in transcriptional functions of nuclear receptors', *Genes and Development*, 14: 121-41.

Grimson, Andrew, Kyle Kai-How Farh, Wendy K. Johnston, Philip Garrett-Engele, Lee P. Lim, and David P. Bartel. 2007. 'MicroRNA Targeting Specificity in Mammals: Determinants beyond Seed Pairing', *Molecular Cell*, 27: 91-105.

Gurianova, Veronika, Dmytro Stroy, Rachele Ciccocioppo, Iveta Gasparova, Daniel Petrovic, Miroslav Soucek, Victor Dosenko, and Peter Kruzliak. 2015. 'Stress response factors as hub-regulators of microRNA biogenesis: implication to the diseased heart: miRNAs and Myocardial Infarction', *Cell Biochemistry and Function*, 33: 509-18.

Hashimoto, Yutaka, Yoshimitsu Akiyama, and Yasuhito Yuasa. 2013. 'Multiple-to-Multiple Relationships between MicroRNAs and Target Genes in Gastric Cancer', *PLoS One*, 8: e62589.

He, Lin, and Gregory J. Hannon. 2004. 'MicroRNAs: small RNAs with a big role in gene regulation', *Nature Reviews Genetics*, 5: 522-31.

He, Xuhua, Lei Shen, Bruno O. Villoutreix, and BjÃ¶rn DahlbÃ¤ck. 1998. 'Amino Acid Residues in Thrombin-sensitive Region and First Epidermal Growth Factor Domain of Vitamin K-dependent Protein S Determining Specificity of the Activated Protein C Cofactor Function', *Journal of Biological Chemistry*, 273: 27449-58.

Heeb, M. J., R. R. Koenen, J. A. Fernández, and T. M. Hackeng. 2004. 'Direct anticoagulant activity of protein S-C4b binding protein complex in Heerlen heterozygotes and normals', *Journal of Thrombosis and Haemostasis*, 2: 1766-73.

Heeb, M. J., R. M. Mesters, G. Tans, J. Rosing, and J. H. Griffin. 1993. 'Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C', *Journal of Biological Chemistry*, 268: 2872-77.

Heeb, Mary J., Jan Rosing, Harry M. Bakker, Jose A. Fernandez, Guido Tans, and John H. Griffin. 1994. 'Protein S Binds to and Inhibits Factor Xa', *Proceedings of the National Academy of Sciences of the United States of America*, 91: 2728-32.

Henry, Jon C., Ana Clara P. Azevedo-Pouly, and Thomas D. Schmittgen. 2011. 'microRNA Replacement Therapy for Cancer', *Pharmaceutical Research*, 28: 3030-42.

Hermeking, H. 2010. 'The miR-34 family in cancer and apoptosis', *Cell Death and Differentiation*, 17: 193-99.

Hurtado, Begona, Xavier Munoz, Maria Carme Mulero, Gemma Navarro, Pere Domenech, Pablo Garcia de Frutos, Merce Perez-Riba, and Nuria Sala. 2008. 'Functional characterization of twelve natural PROS1 mutations associated with anticoagulant protein S deficiency', *Haematologica*, 93: 574-80.

Iannitti, Tommaso, Julio Cesar Morales-Medina, and Beniamino Palmieri. 2014. 'Phosphorothioate oligonucleotides: effectiveness and toxicity', *Current drug targets U6 - ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rfr_id=info%3Asid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=Phosphorothioate+oligonucleotides%3A+effectiveness+and+toxicity&rft.jtitle=Current+drug+targets&rft.au=Iannitti%2C+Tommaso&rft.au=Morales-Medina%2C+Julio+Cesar&rft.au=Palmieri%2C+Beniamino&rft.date=2014-01-01&rft.eissn=1873-5592&rft.volume=15&rft.issue=7&rft.spage=663&rft_id=info%3Apmid%2F24655142&rft_id=info%3Apmid%2F24655142&rft.externalDocID=24655142&paramdict=en-US U7 - Journal Article*, 15: 663.

Incorporated, Long Road Graphics. 2012. 'Activated Partial Thromboplastin Time', Accessed 27 May 2017. http://practical-haemostasis.com/Screening%20Tests/aptt.html

Joshi, Sachindra R., Jared M. McLendon, Brian S. Comer, and William T. Gerthoffer. 2011. "MicroRNAs-Control of Essential Genes: Implications for Pulmonary Vascular Disease." In, 357-64. London, England: SAGE Publications.

Kauppinen, Sakari, Birte Vester, and Jesper Wengel. 2005. 'Locked nucleic acid (LNA): High affinity targeting of RNA for diagnostics and therapeutics', *Drug Discovery Today: Technologies*, 2: 287-90.

Kearney, Phil, Sakari Kauppinen, Marie Lindholm, Henrik Frydenlund Hansen, Steven Gullans, Matthew Lawrence, Peter Sarnow, Urs Berger, Andreas Petri, Ellen Marie Straarup, Sylvia Schütz, Joacim Elmén, Morten Lindow, Susanna Obad, and Maj Hedtjärn. 2008. 'LNA-mediated microRNA silencing in non-human primates', *Nature*, 452: 896-99.

Khan, S., and J. D. Dickerman. 2006. 'Hereditary thrombophilia', *Thromb J*, 4: 15.

Kim, V. Narry, Sung Hee Baek, Jinju Han, Kyu-Hyun Yeom, Minju Kim, Yoontae Lee, and Sanghyuk Lee. 2004. 'MicroRNA genes are transcribed by RNA polymerase II', *The EMBO Journal*, 23: 4051-60.

Kozomara, Ana, and Sam Griffiths-Jones. 2011. 'MiRBase: Integrating microRNA annotation and deep-sequencing data', *Nucleic Acids Research*, 39: D152-D57.

Krishnamurthy, Malathy, Kristina Simon, Anita M. Orendt, and Peter A. Beal. 2007. 'Macrocyclic Helix-Threading Peptides for Targeting RNA', *Angewandte Chemie*, 119: 7174-77.

Kumar, Madhu S., Stefan J. Erkeland, Ryan E. Pester, Cindy Y. Chen, Margaret S. Ebert, Phillip A. Sharp, and Tyler Jacks. 2008. 'Suppression of Non-Small Cell Lung Tumor Development by the Let-7 microRNA Family', *Proceedings of the National Academy of Sciences of the United States of America*, 105: 3903-08.

Kumarswamy, Regalla, Giridhar Mudduluru, Paolo Ceppi, Santoshi Muppala, Miroslaw Kozlowski, Jacek Niklinski, Mauro Papotti, and Heike Allgayer. 2012. 'MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snai1 and is downregulated in non-small cell lung cancer', *International Journal of Cancer*, 130: 2044-53.

Kurreck, Jens. 2003. 'Antisense technologies. Improvement through novel chemical modifications', *European journal of biochemistry / FEBS*, 270: 1628-44.

Kurreck, Jens, Eliza Wyszko, Clemens Gillen, and Volker A. Erdmann. 2002. 'Design of antisense oligonucleotides stabilized by locked nucleic acids', *Nucleic Acids Research*, 30: 1911-18.

Lai, Eric C. 2005. 'miRNAs: Whys and Wherefores of miRNA-Mediated Regulation', *Current Biology*, 15: R458-R60.

Lam, Jenny K. W., Michael Y. T. Chow, Yu Zhang, and Susan W. S. Leung. 2015. 'siRNA Versus miRNA as Therapeutics for Gene Silencing', *Molecular therapy. Nucleic acids*, 4: e252.

Lennox, K. A., and M. A. Behlke. 2011. 'Chemical modification and design of anti-miRNA oligonucleotides', *Gene Therapy*, 18: 1111-20.

Lennox, Kim A., Richard Owczarzy, Derek M. Thomas, Joseph A. Walder, and Mark A. Behlke. 2013. 'Improved Performance of Anti-miRNA Oligonucleotides Using a Novel Non-Nucleotide Modifier', *Molecular therapy. Nucleic acids*, 2: e117.

Liborio-Kimura, T. N., H. M. Jung, and E. K. L. Chan. 2015. 'miR-494 represses HOXA10 expression and inhibits cell proliferation in oral cancer', *ORAL ONCOLOGY*, 51: 151-57.

Ling, H., M. Fabbri, and G. A. Calin. 2013. 'MicroRNAs and other non-coding RNAs as targets for anticancer drug development', *NATURE REVIEWS DRUG DISCOVERY*, 12: 847-65.

Lipe, Brea, and Deborah L. Ornstein. 2011. 'Deficiencies of natural anticoagulants, protein C, protein S, and antithrombin', *Circulation*, 124: e365-e68.

Liu, K. A. I., Songyang Liu, W. E. I. Zhang, Baoxing Jia, Ludong Tan, Z. H. E. Jin, and Yahui Liu. 2015. 'miR-494 promotes cell proliferation, migration and invasion, and increased sorafenib resistance in hepatocellular carcinoma by targeting PTEN', *Oncology Reports*, 34: 1003-10.

Liu, Ning, Svetlana Bezprozvannaya, Andrew H. Williams, Xiaoxia Qi, James A. Richardson, Rhonda Bassel-Duby, and Eric N. Olson. 2008. 'microRNA-133a regulates

cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart', *Genes & development*, 22: 3242-54.

Long, Xiao-Bo, Guang-Bin Sun, Shuang Hu, Geng-Tian Liang, Nan Wang, Xin-Hao Zhang, Ping-Ping Cao, Hong-Tao Zhen, Yong-Hua Cui, and Zheng Liu. 2009. 'Let-7a microrna functions as a potential tumor suppressor in human laryngeal cancer', *Oncology Reports*, 22: 1189-95.

Ma, Y. B., G. X. Li, J. X. Hu, X. Liu, and B. M. Shi. 2015. 'Correlation of miR-494 expression with tumor progression and patient survival in pancreatic cancer', *GENETICS AND MOLECULAR RESEARCH*, 14: 18153-59.

Mac Hale, J. L., N. Nathan, and M. D'Ambra. 1997. 'Intrinsic Anticoagulation: Protein C, Protein S, and Thrombomodulin', *Seminars in Cardiothoracic and Vascular Anesthesia*, 1: 319-32.

Maiti, Mohitosh, Koen Nauwelaerts, and Piet Herdewijn. 2012. 'Pre-microRNA binding aminoglycosides and antitumor drugs as inhibitors of Dicer catalyzed microRNA processing', *Bioorganic & Medicinal Chemistry Letters*, 22: 1709-11.

Maniataki, Elisavet, and Zissimos Mourelatos. 2005. 'A human, ATP-independent, RISC assembly machine fueled by pre-miRNA', *Genes and Development*, 19: 2979-90.

Mattes, Joerg, Ming Yang, and Paul S. Foster. 2007. 'Regulation of MicroRNA by Antagomirs: A New Class of Pharmacological Antagonists for the Specific Regulation of Gene Function?', *American Journal of Respiratory Cell and Molecular Biology*, 36: 8-12.

Maurissen, Lisbeth F. A., M. Christella L. G. D. Thomassen, Gerry A. F. Nicolaes, Björn Dahlbäek, Guido Tans, Jan Rosing, Tilman M. Hackeng, Malmö Clinical Chemistry, fakulteten Medicinska, Medicine Faculty of, Malmö Klinisk kemi, universitet Lunds, Medicine Department of Translational, University Lund, and medicin Institutionen för translationell. 2008. 'Re-evaluation of the role of the protein S-C4b binding protein complex in activated protein C-catalyzed factor Va-inactivation', *Blood*, 111: 3034-41.

McManus, D. D., and J. E. Freedman. 2015. 'MicroRNAs in platelet function and cardiovascular disease', *NATURE REVIEWS CARDIOLOGY*, 12: 711-17.

Melo, Sonia, Alberto Villanueva, Catia Moutinho, Veronica Davalos, Riccardo Spizzo, Cristina Ivan, Simona Rossi, Fernando Setien, Oriol Casanovas, Laia Simo-Riudalbas, Javier Carmona, Jordi Carrere, August Vidal, Alvaro Aytes, Sara Puertas, Santiago Ropero, Raghu Kalluri, Carlo M. Croce, George A. Calin, Manel Esteller, and James E. Dahlberg. 2011. 'Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing', *Proceedings of the National Academy of Sciences of the United States of America*, 108: 4394-99.

Murata, Asako, Takeo Fukuzumi, Shiori Umemoto, and Kazuhiko Nakatani. 2013. 'Xanthone derivatives as potential inhibitors of miRNA processing by human Dicer: Targeting secondary structures of pre-miRNA by small molecules', *Bioorganic and Medicinal Chemistry Letters*, 23: 252-55.

Mustafa, S., I. Pabinger, and C. Mannhalter. 1995. 'Protein S deficiency type I: Identification of point mutations in 9 of 10 families', *Blood*, 86: 3444-51.

Nagalla, Srikanth, Chad Shaw, Xianguo Kong, Altaf A. Kondkar, Leonard C. Edelstein, Lin Ma, Junmei Chen, G. Stanley McKnight, José A. López, Linghai Yang, Ying Jin, Molly S. Bray, Suzanne M. Leal, Jing-Fei Dong, and Paul F. Bray. 2011. 'Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity', *Blood*, 117: 5189-97.

Nahar, Smita, Debojit Bose, Sumit Kumar Panja, Satyen Saha, and Souvik Maiti. 2014. 'Anti-cancer therapeutic potential of quinazoline based small molecules via global

upregulation of miRNAs', *Chemical communications (Cambridge, England)*, 50: 4639.
Nedaeinia, R., M. Sharifi, A. Avan, M. Kazemi, L. Rafiee, M. Ghayour-Mobarhan, and R. Salehi. 2016. 'Locked nucleic acid anti-miR-21 inhibits cell growth and invasive behaviors of a colorectal adenocarcinoma cell line: LNA-anti-miR as a novel approach', *CANCER GENE THERAPY*, 23: 246-53.
Neilson, Joel R., Phillip A. Sharp, and Margaret S. Ebert. 2007. 'MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells', *Nature Methods*, 4: 721-26.
Nesheim, Michael. 2003. 'Thrombin and fibrinolysis', *Chest*, 124: 33S-39S.
Niculescu-Duvaz, Dan, James Heyes, and Caroline Springer. 2003. 'Structure-Activity Relationship in Cationic Lipid Mediated Gene Transfection', *Current Medicinal Chemistry*, 10: 1233-61.
O'Connell, Ryan M., Daniel Kahn, William S. J. Gibson, June L. Round, Rebecca L. Scholz, Aadel A. Chaudhuri, Melissa E. Kahn, Dinesh S. Rao, and David Baltimore. 2010. 'MicroRNA-155 Promotes Autoimmune Inflammation by Enhancing Inflammatory T Cell Development', *Immunity*, 33: 607-19.
Obernosterer, Gregor, Philipp J. F. Leuschner, Mattias Alenius, and Javier Martinez. 2006. 'Post-transcriptional regulation of microRNA expression', *RNA*, 12: 1161-67.
Ohdaira, H., M. Sekiguchi, K. Miyata, and K. Yoshida. 2012. 'MicroRNA-494 suppresses cell proliferation and induces senescence in A549 lung cancer cells', *Cell Proliferation*, 45: 32-38.
Palta, Sanjeev, Richa Saroa, and Anshu Palta. 2014. 'Overview of the coagulation system', *Indian journal of anaesthesia*, 58: 515-23.
Persson, P. B., and M. Mueller. 2015. 'Transcription', *Acta Physiologica*, 215: 159-60.
Pintao, M. C., A. A. Garcia, D. Borgel, M. Alhenc-Gelas, C. A. Spek, de M. C. H. Visser, S. Ganille, and P. H. Reitsma. 2009. 'Gross deletions/duplications in PROS1 are relatively common in point mutation-negative hereditary protein S deficiency', *Human Genetics*, 126: 449-56.
Poy, Matthew N., Jean Hausser, Mirko Trajkovski, Matthias Braun, Stephan Collins, Patrik Rorsman, Mihaela Zavolan, Markus Stoffel, and Harvey F. Lodish. 2009. 'miR-375 Maintains Normal Pancreatic α- and β-Cell Mass', *Proceedings of the National Academy of Sciences of the United States of America*, 106: 5813-18.
Rajeev, Kallanthottathil G., Jan Krützfeldt, Ravi Braich, Markus Stoffel, Nikolaus Rajewsky, Thomas Tuschl, and Muthiah Manoharan. 2005. 'Silencing of microRNAs in vivo with 'antagomirs'', *Nature*, 438: 685-89.
Reid, G., M. E. Pel, M. B. Kirschner, Y. Y. Cheng, N. Mugridge, J. Weiss, M. Williams, C. Wright, J. J. B. Edelman, M. P. Vallely, B. C. McCaughan, S. Klebe, H. Brahmbhatt, J. A. MacDiarmid, and N. van Zandwijk. 2013. 'Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma', *ANNALS OF ONCOLOGY*, 24: 3128-35.
Revel, M., and Y. Groner. 1978. 'Post-Transcriptional and Translational Controls of Gene Expression in Eukaryotes', *Annual Review of Biochemistry*, 47: 1079-126.
Rezende, S. M., R. E. Simmonds, and D. A. Lane. 2004. 'Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex', *Blood*, 103: 1192-201.
Romøren, Kristine, Beate J. Thu, Niels C. Bols, and Øystein Evensen. 2004. 'Transfection efficiency and cytotoxicity of cationic liposomes in salmonid cell lines of hepatocyte and macrophage origin', *BBA - Biomembranes*, 1663: 127-34.
Roush, Sarah, and Frank J. Slack. 2008. 'The let-7 family of microRNAs', *Trends in Cell Biology*, 18: 505-16.

Schmidt, M. F. 2014. 'Drug target miRNAs: chances and challenges', *TRENDS IN BIOTECHNOLOGY*, 32: 578-85.
Schöler, Hans R., Matthias Wilmanns, and Attila Reményi. 2004. 'Combinatorial control of gene expression', *Nature Structural & Molecular Biology*, 11: 812-15.
Shahar, Tal, Avital Granit, Daniel Zrihan, Tamar Canello, Hanna Charbit, Ofira Einstein, Uri Rozovski, Sharona Elgavish, Zvi Ram, Tali Siegal, and Iris Lavon. 2016. 'Expression level of miRNAs on chromosome 14q32.31 region correlates with tumor aggressiveness and survival of glioblastoma patients', *Journal of Neuro-Oncology*, 130: 413-22.
Shimono, Yohei, Maider Zabala, Robert W. Cho, Neethan Lobo, Piero Dalerba, Dalong Qian, Maximilian Diehn, Huiping Liu, Sarita P. Panula, Eric Chiao, Frederick M. Dirbas, George Somlo, Renee A. Reijo Pera, Kaiqin Lao, and Michael F. Clarke. 2009. 'Downregulation of miRNA-200c Links Breast Cancer Stem Cells with Normal Stem Cells', *Cell*, 138: 592-603.
Singh, Yashveer, Pierre Murat, and Eric Defrancq. 2010. 'Recent developments in oligonucleotide conjugation', *Chemical Society reviews*, 39: 254-27.
Subramanian, Nithya, Jagat R. Kanwar, Rupinder K. Kanwar, and Subramanian Krishnakumar. 2015. 'Blocking the Maturation of OncomiRNAs Using pri-miRNA-17∼92 Aptamer in Retinoblastoma', *nucleic acid therapeutics*, 25: 47-52.
Summerton, J. 2005. 'Morpholino Antisense Oligos: Applications in Biopharmaceutical Research', *Innovations in Pharmaceutical Technology*, 17: 32-37.
Summerton, James E. 2003. 'Morpholinos and PNAs compared', *International Journal of Peptide Research and Therapeutics*, 10: 215-36.
Sun, Hai-Bing, X. I. Chen, Hong Ji, T. A. O. Wu, Hong-Wei Lu, Y. A. N. Zhang, H. U. A. Li, and Yi-Ming Li. 2014. 'miR-494 is an independent prognostic factor and promotes cell migration and invasion in colorectal cancer by directly targeting PTEN', *International Journal of Oncology*, 45: 2486-94.
Szulwach, Keith E., Zain Paroo, Wendi Li, Claes Wahlestedt, Ranhui Duan, Lianghua Lu, Ge Shan, Chuan He, Peng Jin, Qinghua Liu, Ahmad M. Khalil, Mohammad A. Faghihi, Junliang Zhang, Zhangjie Shi, Anthony W. S. Chan, and Yujing Li. 2008. 'A small molecule enhances RNA interference and promotes microRNA processing', *NATURE BIOTECHNOLOGY*, 26: 933-40.
Taniguchi, F., E. Morishita, A. Sekiya, H. Nomoto, S. Katsu, S. Kaneko, H. Asakura, and S. Ohtake. 2016. 'Gene analysis of six cases of congenital protein S deficiency and functional analysis of protein S mutations (A139V, C449F, R451Q, C475F, A525V and D599TfsTer13)', *Thromb Res*, 151: 8-16.
Tay, J., J. Tiao, Q. Hughes, G. Gilmore, and R. Baker. 2016. 'Therapeutic Potential of miR-494 in Thrombosis and Other Diseases: A Review', *AUSTRALIAN JOURNAL OF CHEMISTRY*, 69: 1078-93.
Tay, J. W., G. Romeo, Q. W. Hughes, and R. I. Baker. 2013. 'Micro-ribonucleic Acid 494 regulation of protein S expression', *J Thromb Haemost*, 11: 1547-55.
ten Kate, M. K., and J. Van der Meer. 2008. 'Protein S deficiency: a clinical perspective', *Haemophilia*, 14: 1222-8.
Teruel-Montoya, R., F. R. Rosendaal, and C. Martinez. 2015. 'MicroRNAs in hemostasis', *J Thromb Haemost*, 13: 170-81.
Teruel, Raúl, Javier Corral, Virginia Pérez-Andreu, Irene Martínez-Martínez, Vicente Vicente, and Constantino Martínez. 2011. 'Potential role of miRNAs in developmental haemostasis', *PLoS One*, 6: e17648.
Van De Poel, Robbert H. L., Joost C. M. Meijers, and Bonno N. Bouma. 1999. 'Interaction between protein S and complement C4b-binding protein (C4BP). Affinity studies

using chimeras containing C4BP β-chain short consensus repeats', *Journal of Biological Chemistry*, 274: 15144-50.

van der Meer, J. H. M., T. van der Poll, and C. van 't Veer. 2014. 'TAM receptors, Gas6, and protein S: roles in inflammation and hemostasis', *Blood*, 123: 2460-69.

van Rooij, Eva, Daniel Quiat, Brett A. Johnson, Lillian B. Sutherland, Xiaoxia Qi, James A. Richardson, Robert J. Kelm, and Eric N. Olson. 2009. 'A Family of microRNAs Encoded by Myosin Genes Governs Myosin Expression and Muscle Performance', *Developmental Cell*, 17: 662-73.

Ventura, Andrea, Amanda G. Young, Monte M. Winslow, Laura Lintault, Alex Meissner, Stefan J. Erkeland, Jamie Newman, Roderick T. Bronson, Denise Crowley, James R. Stone, Rudolf Jaenisch, Phillip A. Sharp, and Tyler Jacks. 2008. 'Targeted Deletion Reveals Essential and Overlapping Functions of the miR-17∼92 Family of miRNA Clusters', *Cell*, 132: 875-86.

Vigano-D'Angelo, S., A. D'Angelo, C. E. Kaufman Jr, C. Sholer, C. T. Esmon, and P. C. Comp. 1987. 'Protein S deficiency occurs in the nephrotic syndrome', *Annals of Internal Medicine*, 107: 42-47.

Wada, Fumito, Shunsuke Wada, Hidenori Yasuhara, Motoki Sawamura, Reiko Waki, Tsuyoshi Yamamoto, Mariko Harada-Shiba, and Satoshi Obika. 2016. 'Evaluation of the effects of chemically different linkers on hepatic accumulations, cell tropism and gene silencing ability of cholesterol-conjugated antisense oligonucleotides', *Journal of Controlled Release*, 226: 57-65.

Wang, Xiaohong, Xiaowei Zhang, Xiao-Ping Ren, Jing Chen, Hongzhu Liu, Junqi Yang, Mario Medvedovic, Zhuowei Hu, and Guo-Chang Fan. 2010. 'MicroRNA-494 targeting both proapoptotic and antiapoptotic proteins protects against ischemia/reperfusion-induced cardiac injury', *Circulation*, 122: 1308-18.

Welten, Sabine M. J., Antonius J. N. M. Bastiaansen, Rob C. M. de Jong, Margreet R. de Vries, Erna A. B. Peters, Martin C. Boonstra, Søren P. Sheikh, Nicola La Monica, Ekambar R. Kandimalla, Paul H. A. Quax, and A. Yaël Nossent. 2014. 'Inhibition of 14q32 MicroRNAs miR-329, miR-487b, miR-494, and miR-495 Increases Neovascularization and Blood Flow Recovery After Ischemia', *Circulation Research*, 115: 696-708.

Williams, Andrew H., Gregorio Valdez, Viviana Moresi, Xiaoxia Qi, John McAnally, Jeffrey L. Elliott, Rhonda Bassel-Duby, Joshua R. Sanes, and Eric N. Olson. 2009. 'MicroRNA-206 Delays ALS Progression and Promotes Regeneration of Neuromuscular Synapses in Mice', *Science*, 326: 1549-54.

Winkler, Johannes. 2013. 'Oligonucleotide conjugates for therapeutic applications', *Therapeutic delivery*, 4: 791.

Wu, J., and J. Bag. 1998. 'Negative Control of the Poly(A)-binding Protein mRNA Translation Is Mediated by the Adenine-rich Region of Its 5*-Untranslated Region', *The Journal of Biological Chemistry*, 273: 34535 - 42.

Yamamoto, Hirotaka, Katsutaro Morino, Yoshihiko Nishio, Satoshi Ugi, Takeshi Yoshizaki, Atsunori Kashiwagi, and Hiroshi Maegawa. 2012. 'MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead box j3', *American Journal of Physiology - Endocrinology And Metabolism*, 303: 1419-27.

Yeung, Man Lung, and Kuan-Teh Jeang. 2011. 'MicroRNAs and Cancer Therapeutics', *Pharmaceutical Research*, 28: 3043-49.

Zabner, Joseph. 1997. 'Cationic lipids used in gene transfer', *Advanced Drug Delivery Reviews*, 27: 17-28.

Zhao, X. Q., T. J. Liang, and J. W. Fu. 2016. 'miR-494 inhibits invasion and proliferation of gastric cancer by targeting IGF-1R', *EUROPEAN REVIEW FOR MEDICAL AND PHARMACOLOGICAL SCIENCES*, 20: 3818-24.

Appendix

A. Recipes

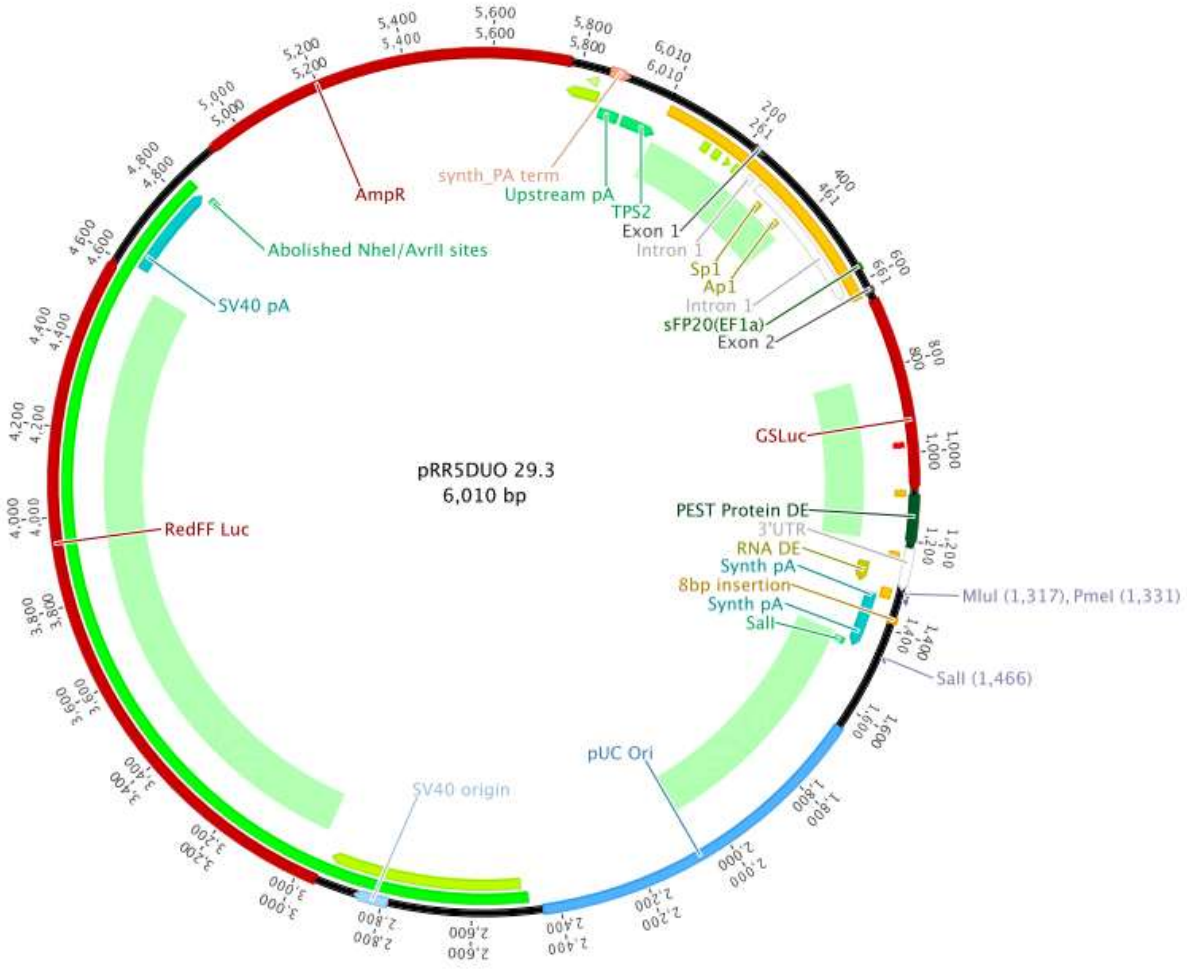
DMEM medium
500mL Bottle of DMEM medium
50mL Foetal calf serum
5mL Penicillin/ Streptomycin
5mL Non-essential amino acids
5mL Sodium pyruvate

LB Broth/ Media
800mL Milli-Q Water
10g Bacto-tryptone
5g Yeast
10g NaCl
pH is adjusted to 7.5 using NAOH

1% Agarose Gel
4.0g agarose
400mL 1x TAE

1x Lysis buffer
30µl 5x lysis buffer stock
120µl Sigma Water

B. pRR5-Duo-37 vector construct



C. *PROS1* sequence including 3'UTR inserted into plasmid

```
ORIGIN
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       61 ctcagcagtg tttactaggc ctccaacact agagcccatc ccccagctcc gaaaagcttc
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      541 aagccaggga ggtctttgaa aatgacccgg aaacggatta tttttatcca aaatacttag
      601 tttgtcttcg ctcttttcaa actgggttat tcactgctgc acgtcagtca actaatgctt
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      961 tgaagccaag catttgtggc acagctgtgt gcaagaacat cccaggagat tttgaatgtg
     1021 aatgccccga aggctacaga tataatctca aatcaaagtc ttgtgaagat atagatgaat
     1081 gctctgagaa catgtgtgct cagctttgtg tcaattaccc tggaggttac acttgctatt
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     1981 cacaggatat tctgttatct gttgaaaata ctgtaatata tcggatacag gccctaagtc
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     2161 tggacaaagc aatgaaagca aaagtggcca catacctggg tggccttcca gatgttccat
     2221 tcagtgccac accagtgaat gccttttata atggctgcat ggaagtgaat attaatggtg
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//
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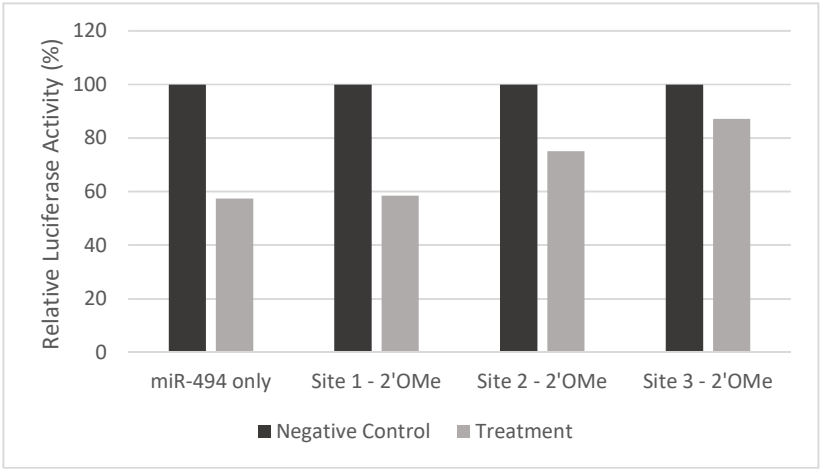
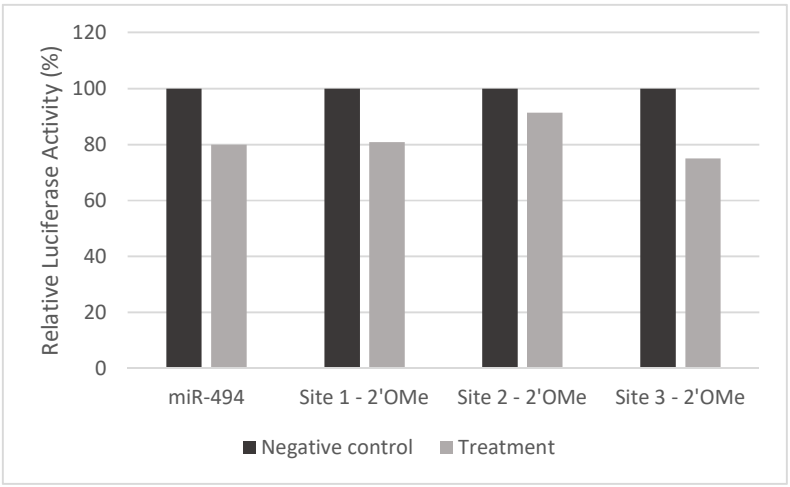
D. Luciferase Assay Independent results

A.



B.



C.

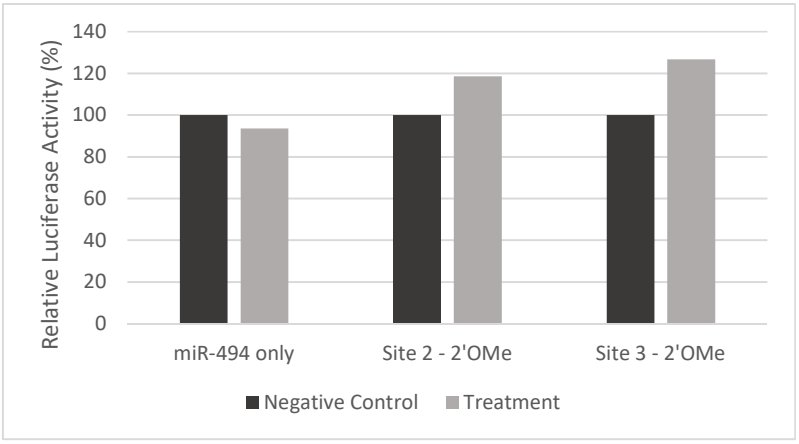


Figure Appx 1. Initial independent result of HuH-7 cells transfected with 50nM miR-494 and 50nM of 2'OMe ASOs specific to the 3 sites found of *PROS1* 3'UTR. **A.** - **B.** 2'OMe oligonucleotides at sites 1, 2 and 3. **C.** 2'OMe oligonucleotides are sites 2 and 3.